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Targeting Biofilm-Forming *Serratia marcescens* by Bacteriophages and Disrupting Biofilm and Exopolysaccharide Production

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Abstract

Objective: This study aimed to isolate two phages from soil and wastewater to assess their effectiveness in reducing biofilm and EPS production and disrupting biofilms in antibiotic-resistant *S. marcescens*.

Methods: Soil and wastewater samples were added to a broth medium to allow phage infection of the bacterial host and enhance phage isolation. Bacteriophage titers, stability at different pH values and temperature, and host spectrum were detected. The effect of the combination of phage and antibiotics on the target host was recorded. Also, the inhibition of biofilm and EPS formation by the presence of phages was studied.

Results: Two phages were obtained from wastewater and soil. Plaque morphology analysis revealed distinct characteristics for each bacteriophage. Both sources yielded high titers of phages specific to *S. marcescens*, with the wastewater-derived phage (*S.wph*) showing a slightly higher titer (1.67×10^9 PFU/ml) compared to the soil-derived phage (*S.So.ph*). The stability and host range of *S.wph* and *S.So.ph* were studied. The potential of *S.wph* to target multiple bacterial species could be valuable in developing phage-based therapies against diverse bacterial infections. The recorded MICs were 10^5 and 10^6 PFU/ml for *S.wph* and *S.So.ph* targeting *S. marcescens*, respectively. Ampicillin exhibited minimal inhibitory effects on *S. marcescens* with bacterial growth, while the combination of ampicillin with *S.wph* maintained the strongest antibacterial effect with no significant differences to *S.wph* monotherapy. In contrast, the ampicillin and *S.So.ph* combination displayed a moderate bacterial inhibition compared to ampicillin with *S.wph,* but there is a considerable difference compared to *S.So.ph* alone. Wastewater-derived phage (*S.wph*), soil-derived phage (*S.So.ph*), and their mixture inhibited biofilm formation and EPS secretion by *S. marcescens*.

Conclusion: This research underscores the promising potential of bacteriophages as alternative treatments to conventional antibiotics, particularly in the face of increasing bacterial antibiotic resistance.

Keywords: Bacteriophages, antibiotic-resistant, *Serratia marcescens* exopolysaccharide, biofilms

Introduction

Serratia marcescens is a genus of Gram-negative bacteria known for producing a red pigment called prodigiosin. It can cause severe infections, particularly in immunocompromised patients, and exhibits adaptability to various environments.[1,2] *S. marcescens* can thrive in diverse habitats, including water, soil, and animals, which has made it a significant focus of many studies. This bacterium form complex aggregates of cells known as biofilms, which are surrounded by high-molecular-weight compounds called EPS that facilitate attachment to surfaces[3–6] and played a crucial role in the stability of biofilms and protection from various environmental factors and antibiotics which increase antimicrobial resistance and posing a major challenge in treating bacterial infections.[7–9] The emergence of antibiotic resistance complicates medical treatment and raises the risk of chronic infections. In this context, bacteriophages have emerged as a promising alternative for combating resistant bacteria.

Bacteriophages specifically infect bacteria and secrete some enzymes that can disrupt biofilms and reduce the production of EPS.[9–11] These viruses can be utilized in phage therapy as an alternative to traditional antibiotics, especially against resistant strains.[12,13] Recent research has explored the isolation of bacteriophages from various sources, highlighting their significant potential in multiple fields. Mangieri (2021)[14] focused on isolating bacteriophages from wastewater using *Escherichia coli* and studied the role of bacteriophages as natural bacterial predators. In another study, Pan et al. (2023)[15] obtained bacteriophages from soil and used them as biocontrol agents for *Pseudomonas aeruginosa* in contaminated areas. Similarly, Zafar et al. (2024)[16] investigated the isolation of bacteriophages targeting antibiotic-resistant *Staphylococcus aureus* from hospital environments using enrichment culture and plaque assay methods. The potential of bacteriophages in treating hospital-acquired infections and to control *Salmonella enterica* in agriculture were confirmed.[17,18]

Bacteriophage combinations that target various bacterial strains have emerged as a promising approach for combating bacterial infections. Yang et al. (2020)[19] utilized a bacteriophage cocktail to target multidrug-resistant *P. aeruginosa* and reduced bacterial load *in vitro* and *in vivo*. Similarly, Mickos et al. (2023)[20] explored the application of a bacteriophage cocktail to control *E. coli* and *S. enterica* in food processing environments. The phage cocktail significantly reduced bacterial contamination on surfaces and reduced *S. aureus* in dairy products and *Salmonella* and *Campylobacter* contamination in poultry products,[21,22] while Byun et al. (2023)[23] employed a cocktail of bacteriophages to treat *Listeria monocytogenes* during food processing and storage. The synergistic effect of the bacteriophage with the antibiotic ciprofloxacin in treating *P. aeruginosa* infections[24] and between bacteriophages and ampicillin against *E. coli* associated with urinary tract

infections.[25] Phages improve the antibacterial effects of Vancomycin against MRSA infections and prevent the emergence of resistant strains,[25,26] while bacteriophage cocktail combined with tetracycline is used for treating *S. enterica* infections.[27] Furthermore, Rastegar et al. (2024)[28] explored the use of bacteriophages in combination with colistin to treat *Acinetobacter baumannii* infections and effectively reduce bacterial load.

In recent years, bacteriophages and antibiotics have garnered attention as a potential strategy to combat multidrug-resistant bacteria, and more research is needed. The aim of this study is to evaluate the effectiveness of treating *S. marcescens* with individual phages, phage cocktail, both alone and in combination with an antibiotic, and investigate these treatments as alternative solutions for addressing antibiotic-resistant bacteria and biofilms.

Materials and Methods

Bacteriophage Isolation and Enrichment from Environmental Samples

Bacteriophages were isolated from two sources in Saudi Arabia during the summer of 2023 for characterization and analysis. One of the samples collected was sewage water from the Jeddah Wastewater Treatment Plant, Al Faiasalia, western region of Jeddah. This plant receives effluent from several sources, including the King Faisal Specialist Hospital and Research Center. In addition to the sewage sample, a soil sample was taken from a farm in Al-Medina Al-Munawwara (coordinates: 24°41'07.4"N 39°17'16.2"E) and stored at 4°C before processing. The protocol for enrichment and isolation involved combining 10 mL of the wastewater sample with 10 mL of double-strength Luria-Bertani broth (2X LB), 200 µl of 2 mM calcium chloride ($CaCl_2$), and 10 mL of an overnight bacterial culture (from 10^6 CFU/ml). This mixture was incubated at 37°C for 24 hours with agitation at 120 rpm. After incubation, the culture was centrifuged at 6000 rpm at 4°C for 10 minutes to remove large particulates. The resulting supernatant was then filtered sequentially through 0.45 µm and 0.22 µm membrane filters (Millex™ Sterile Syringe Filters) to eliminate the residual bacterial cells. An agar overlay assay was subsequently performed to determine phage concentrations (PFU/ml). The bacteriophage preparation underwent a series of purification steps, which included three successive rounds of individual plaque isolation. After these steps, the phages were amplified using their respective bacterial host strains to generate the final purified lysate. Colonies were counted, ensuring the total was between 30 and 300 colonies, and the phage titer was expressed as plaque-forming units (PFU/ml) as reported by Elahi et al. (2021), Artawinata et al. (2023), and Khan Mirzaei and Nilsson (2015),[29–31] according to the formula:

$$\textbf{PFU/ml} = \textbf{Number of plaques} \times \textbf{Dilution factor}$$

Characterization of Phages

Assessment of Plaque Morphology

The characteristics of the plaques were documented by examining their dimensions, peripheral features, and demarcation patterns. This assessment involved evaluating the plaques' diameter, the nature of their borders (such as clear-cut or diffuse), and the overall shape and appearance of the plaque boundaries. These detailed observations of plaque morphology provide valuable insights into the interactions between bacteriophages and their host bacteria.[32,33]

Bacteriophage Titer Determination

The titer of the isolated phages was determined using the double-layer agar method to estimate the concentration of infectious phage particles.[34]

Bacteriophage Stability under Various Physical Conditions

The research examined the impact of diverse environmental factors on bacteriophage viability. Specifically, it investigated the effects of pH variations and temperature fluctuations on phage stability. The double-layer agar technique was employed to quantify changes in phage viability under these different conditions. The decrease in phage activity or survival was meticulously documented, providing a comprehensive understanding of the phages' resilience to various physical stressors.[35,36]

Host Spectrum

A spot assay was performed to determine the host range of the lytic phages infecting *S. marcescens*. This was done using seven bacterial strains, namely *E. coli* (8739), *E. coli* (25922), *E. coli* (23355), *Klebsiella pneumonia* (ESBL) (700613), *K. pneumonia* (60603), *Proteus mirabilis* (12453), *S. marcescens* (13880), and *S. typhi* (14028) were obtained from the King Abdulaziz University Hospitals. The results were determined after pipetting 5 µl droplets of 10^7 PFU/ml onto the growth of different bacterial strains prepared on LB plates. The formation of lysis plaques at 37°C is considered a positive result.[37]

Determining the MIC of Phages

The MICs of phages were determined using the broth dilution method in a 96-well microplate. The test bacterium was grown overnight on Nutrient agar, and individual colonies picked from the plates were suspended in TSB to match the 0.5 McFarland standard (1.5×10^8 CFU/ml), then distributed in 100 µl volumes into a 96-well microtiter plate. The phage preparations were diluted to contain different numbers of phages, ranging from ~10^9 to 10^3 PFU, and 100 µl of phage dilutions were added to wells containing bacterial cells. Corresponding cell controls, phage controls, and media controls were maintained. The microtiter plates were incubated at 37°C overnight without shaking. Finally, 5 µl of the indicator Resazurin solution was added for 2 to 4 hours.[38] The lowest phage concentration at which no growth was seen was regarded as the MIC.

Resistance Pattern and Biofilm Formation of S. marcescens

In this study, *S. marcescens* (ATCC-13880), obtained from the King Abdulaziz University Hospital (KAUH), Saudi Arabia, was used as the test organism for phage isolation. Vitek 2 Identification System (VITEK® 2: Healthcare - BIOMERIEUX) was used to confirm the identification and determine the resistant pattern.[39] The ability to form biofilm was detected, according to Chibeu et al. (2009).[40] In a sterile test tube, 200 µl of the *S. marcescens* suspension (approximately 0.5 nm, 0.10^6 CFU/ml) was added to 5 ml of Tryptic Soy Broth (TSB) medium supplemented with 1% glucose. The test tubes were then incubated at 37°C for 24 hours. After the incubation period, the

tube contents were drained, and the tube was washed with phosphate-buffered saline (PBS) solution (pH 7.4) and left to air dry. Next, 1 ml of a 0.1% w/v crystal violet stain was added to the tube and allowed to sit for 15 minutes. Subsequently, the tube was washed with PBS solution. The presence of biofilms was assessed by observing the purple coloration on the walls of the tube, which could be dissolved using acetic acid and measured by absorbance.

Effect of Bacteriophage and Antibiotics on Bacterial Growth

Our focus was on targeting antibiotic-resistant bacteria before biofilm formation. The inoculum of *S. marcescens* was prepared from a 24-hour TSB culture, and the suspension was adjusted to 0.5 McFarland turbidity standards (1.5×10^8 CFU/ml). Ampicillin (Merck Life Science, Darmstadt, Germany) and phages with titer 1×10^7 were prepared in TSB. A series of two-fold antibiotic dilutions in TSB containing phages were made in a 96-well plate, and 100 µl of bacterial suspension was added to the wells. Additionally, controls of the sterility of antibiotics and phages were prepared. *S. marcescens* growth in TSB (without killing agents). Also, the effects of single agents (phage or antibiotic) on *S. marcescens* growth were tested. The plates were incubated for 48 hrs at 37°C, and the growth was measured by absorbance at 600 nm using a Multiskan GO reader (Thermo Fisher Scientific, Vantaa, Finland) as described before.[41,42]

Effect of Bacteriophage on Biofilm using Microtiter Plate Assay

The prepared cultures of *S. marcescens* (50 µl, OD_{600} of 0.05) was mixed, with or without phage (final concentration, 10^7 PFU/ml), and TSB was added up to100-µl final volume in each well. The mixture was plated in the wells of round-bottomed 96-well microtiter plates and incubated without shaking at 37°C to allow biofilm formation. The obtained biofilms stained with crystal violet were performed.[38,43] Briefly, the medium inside the wells of a 96-well microtiter plate was removed, and the plate was washed with water and air dried for 20 min. Subsequently, 125 µl 0.1% crystal violet solution was added for 10 min. to the wells to stain the biofilms, which were washed three times with Milli-Q water and dried for 20 min. For dissolving the stained crystal violet, 125 µl 30% acetic acid was added and 100 µl of the solubilized crystal violet in each well was transferred to a flat-bottomed 96-well microtiter plate, and OD_{600} was measured using a microplate spectrometer.[44]

$$\% \text{ biofilm inhibition} = \left(\text{OD control} - \frac{\text{OD test}}{\text{OD control}} \right) \times 100$$

OD: optical density.

The Effect of Phages on Exopolysaccharides Content in Serratia marcescens Cells

The impact of phages on EPS formation in target bacteria was examined. The sample preparation involved adding 2.5 ml of double-concentrated tryptic soy broth (2X TSB), 0.5 ml of *S. marcescens*, and 2 ml of either *S.wph* or *S.So.ph*, individually purified phages. The effect of a 1:1 mixture of the two phages was also tested. All samples were incubated for 24 hours. Following the incubation, bacterial cells were removed by centrifugation, the supernatant was lyophilized, and EPS was extracted using the method described by Smitinont et al. (1999).[45] Overnight cultures of *S. marcescens* were centrifuged at 10,000 rpm for 20 minutes at 4°C to separate the cells from the EPS produced by the bacteria. The supernatant was transferred to a new tube and mixed with two volumes of chilled absolute ethanol, then incubated for 24 hours at 4°C to precipitate the EPS. The precipitated EPS was collected by centrifugation under the same conditions, and the supernatant was discarded. The resulting EPS pellet was dried at room temperature, and the carbohydrate content was determined using the phenol-sulfuric acid method[46,47] with a spectrophotometer. Approximately 0.05 mg of the EPS pellet was dissolved in 0.5 ml of deionized water from both the control and phage-treated samples. The total extracellular carbohydrate content in the EPS was quantified using the phenol-sulfuric acid method with a spectrophotometer.[45,46] The experiment was repeated three times to ensure accurate measurements.

The quantity was measured using a glucose standard curve (100–1000 mg/l). A control sample containing only phenol and sulfuric acid was also measured for comparison. The standard curve and sample analyses were used to calculate the total EPS content (µg/mg of cells), allowing for comparisons between the tested bacteria and the control.[46,47] The untreated EPS served as a baseline to compare the effects of phage treatment on EPS characteristics. Biofilm inhibition percentage was estimated using the following formula:

Activity = Standard concentration (mg/l)/Concentration of the substance (mg/l)
Inhibition = (1−Activity Without Inhibitor/ Activity with Inhibitor) × 100%

Statistical Analysis

The information was captured as averages ± SE from three separate experiments. A one and two-way analysis of variance (ANOVA) were used to compare the results. GraphPad Prism software version 10.4.1(627) for data analyses was used to detect any significant differences between sample and control at $P < 0.05$.

Results

Bacteriophage Isolation from Environmental Samples and Plaque Morphology Assessment

From wastewater and soil samples, the isolation of two bacteriophages, *S.wph* from wastewater and *S.So.ph* from soil, targeting *S. marcescens*, was successfully achieved. Plaque morphology analysis revealed distinct characteristics for each bacteriophage. *S.wph* produced small, clear plaques without a halo, while *S.So.ph* formed plaques of varying sizes, ranging from small to large, with most exhibiting clear, clean appearances. The *S.So.ph* plaques uniquely displayed a halo formation, distinguishing them from the other phages, as shown in Fig. 1A and B.

The results presented in Table 1 provide valuable insights into the isolation and quantification of bacteriophages from two distinct environmental sources: wastewater and soil. Both sources yielded high titers of phages specific to *S. marcescens*, with the wastewater-derived phage (*S.wph*) showing a slightly

higher titer (1.67×10^9 PFU/ml) compared to the soil-derived phage (*S.So.ph*), which was 1.39×10^9 PFU/ml.

Evaluation of Bacteriophage Stability under Various Physical Conditions

The stability analysis of *S. marcescens* bacteriophages, *S.wph and S.So.ph,* under various pH and temperature conditions reveals important characteristics for their potential applications. Table 2 showed that both phages exhibit optimal stability at neutral pH (7), maintaining high activity, while showing moderate stability in alkaline conditions (pH 10–12). However, their instability in acidic environments (pH 2–4) suggests limitations for applications in low pH settings. Regarding thermal stability, both phages demonstrate remarkable resilience across a wide temperature range, with very high stability at 7°C, indicating optimal storage conditions. *S.So.ph* shows superior stability at refrigeration (4°C) and high temperatures (70°C) compared to *S.wph*, while both maintain some activity even at extreme temperatures (–80°C and 80°C), as shown in Table 2.

Host Spectrum

The host range analysis of bacteriophages *S.wph* and *S.So.ph* reveals distinct infectivity patterns. *S.wph* demonstrates a broad host range, successfully infecting all six tested bacterial strains across different genera, including *E. coli, Klebsiella, Proteus, Serratia,* and *Salmonella*. In contrast, *S.So.ph* exhibits a narrower specificity, lysing only *E. coli* 23355 and *S. marcescens*. Both phages effectively target their presumed primary host, *S. marcescens* (Table 3).

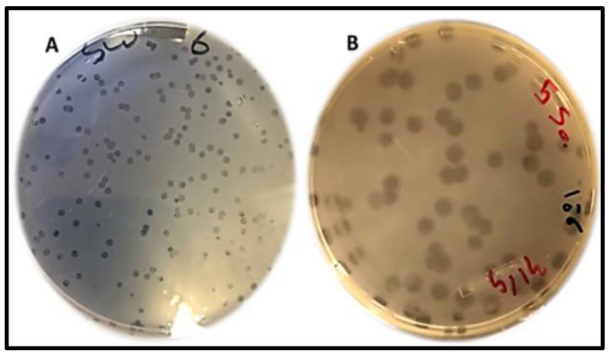

Fig. 1 **Bacteriophage clear zones lysis (plaques) of the two bacteriophage on TSB medium, A: S.wph and B: S.So.ph.**

Bacterial Strain and Resistance to Antibiotic

The tested bacterium *S. marcescens* was selected as a test bacterium and grown in LB and MHA media (Figure 2). It showed good growth in both media. The sensitivity to different antibiotics was detected and the Vitek 2 Identification System (Fig. 2C and Table 4). It was resistant to 9 out of 15 tested antibiotics.

Minimum Inhibitory Concentration of the two Tested Bacteriophages Targeting S. marcescens

The MIC of bacteriophages refers to the lowest concentration of phages required to inhibit bacterial growth. The recorded MICs were 10^5 and 10^6 PFU/ml for *S.wph* and *S.So.ph* targeting *S. marcescens*, respectively (Figure 3).

Effect of Bacteriophage and Antibiotics on Bacterial Growth

The data presented in Figure 4 and Table 5 illustrated the comparative efficacy of bacteriophages *S.wph* and *S.So.ph*, alongside ampicillin, in inhibiting *S. marcescens* growth over 48 hours measured by both optical density and color change from blue to pink after bacterial growth. The results reveal a marked superiority of bacteriophage *S.wph* in bacterial growth suppression compared to *S.So.ph* and ampicillin monotherapy. Bacteriophage *S.wph* demonstrated rapid and sustained antibacterial activity, reducing bacterial density (OD_{590}) from 0.50 to 0.02 within 24 hours. In contrast, ampicillin exhibited minimal inhibitory effects, with bacterial growth patterns closely mirroring untreated control. Notably, the combination of ampicillin with *S.wph* maintained the strong antibacterial effect with no significant differences to *S.wph* monotherapy, while the ampicillin and *S.So.ph* combination displayed a moderate bacterial inhibition compared to ampicillin with *S.wph*.

Table 1. **Measured of the bacteriophage titers of *S.wph* and *S.So.ph***

Sample source	Isolated phage	Bacteria conc. CFU/ml	Phage titer PFU/ml	Dilution
Wastewater	*S.wph*	1.5×10^8	1.67×10^9	167×10^{-5}
Soil	*S.So.ph*	1.5×10^8	1.39×10^9	139×10^{-5}

Table 2. **Stability of bacteriophages targeting *S. marcescens* in different pH values and temperatures**

pH value	*S. marcescens*		Temperature ˚C	*S. marcescens*	
	Phage I (*S.wph*)	Phage II (*S.So.ph*)		Phage I (*S.wph*)	Phage II (*S.So.ph*)
2	–	–	–80	+	++
4	–	–	4	++	+++
7	+++	+++	7	++++	++++
10	++	++	70	++	+++
12	++	++	80	++	++

++++: Very High stability (> 90% of bacteriophages remain active), +++ : High stability (90–70%), ++: Moderate stability (70–40%), +: Low stability (10–40%), and – : Unstable.

Table 3. **The host range of the two bacteriophages tested against some bacteria**

Tested bacteria	Isolated phage for *Serratia*	
	S.wph	***S.So.ph***
E. coli (25922)	+	–
E. coli (23355)	+	+
Klebsiella pneumonia (700613)	+	–
Proteus mirabilis (12453)	+	–
Serratia marcescens (13880)	+	+
Salmonella typhi (14028)	+	–

+: A positive result and successful outcomes in the spot assay method, -: a negative result and unsuccessful outcomes in the assay.

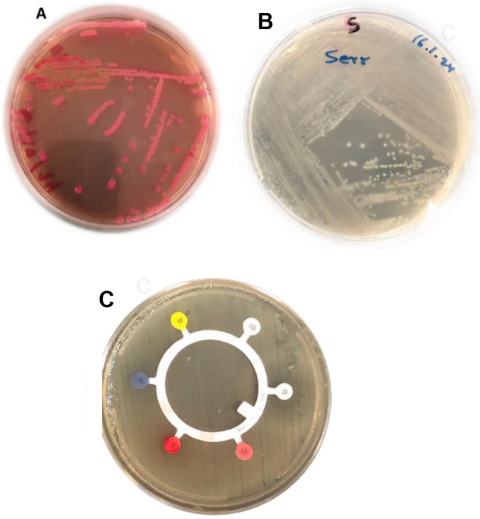

Fig. 2 ***S. marcescens*** **on LB (A), on MHA (B) and sensitivity to antibiotics on MHA (C).**

Table 4. **Resistance of *S. marcescens* to different antibiotics detected by Vitek2**

SN.	Antibiotic	MIC	Results
1	Amoxicillin/Clavulanic Acid	≥32	R
2	Cefazolin	≥64	R
3	Cefuroxime	≥64	R
4	Cefuroxime Axetil	≥64	R
5	Ceftazidime	≥64	R
6	Ceftriaxone	32	R
7	Cefepime	≥0.12	S
8	Ertapenem	≥0.12	S
9	Imipenem	0.5	S
10	Meropenem	4	R
11	Amikacin	4	R
12	Gentamicin	≤1	S
13	Ciprofloxacin	≤0.06	S
14	Nitrofurantoin	256	R
15	Trimethoprim/Sulfamethoxazole	≤20	S

S: Sensitive; R: Resistance.

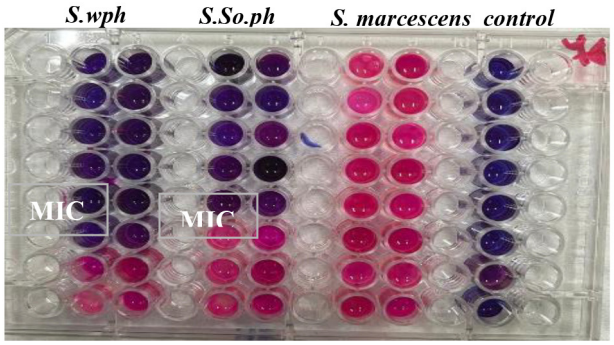

Fig. 3 **Minimum Inhibitory Concentration (MIC) of the tested bacteriophages, *S.wph* and *S.So.ph* for *S. marcescens*.**

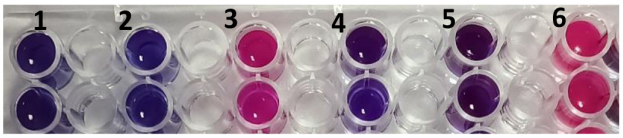

Fig. 4 **Biocontrol method of phages and antibiotic using *S. marcescens* as test bacterium: (1) *S.wph* + Ampicillin, (2) *S.So.ph* + Ampicillin, (3) Ampicillin, (4) *S.wph*, (5) *S.So.ph*, and (6) *S. marcescens*.**

Biofilm Formation by S. marcescens and Confirmation of its Disturbance by Bacteriophage

The tube adherence assay for biofilm formation yielded positive results for *S. marcescens*. Upon completion of the protocol, distinct, purple-stained biofilm matrices were observed adhering to the inner surface of the glass tubes compared to control (without bacteria). The control recorded no color (Fig 5A) while the intensity and distribution of the purple coloration varied slightly between replicates, which is consistent with the inherent biological variability in biofilm formation in a tube or in microtiter plate (Figure 5B and C).

The study investigated the effect of phages isolated from two different sources, *S.wph* from wastewater and *S.So.ph* from soil, on the biofilm-forming bacteria *S. marcescens*. The results of biofilm absorption (OD_{490} nm) revealed that the phage isolated from wastewater (*S.wph*) was more effective in reducing biofilm absorption compared to the phage isolated from soil (*S.So.ph*). All the tested phages had the ability to inhibit biofilm formation up to 24 hrs, then biofilm formation increased by time (Figure 6). Bacteriophage *S.wph* demonstrated good efficacy, achieving an inhibition rate of 53.58%, indicating a moderate capacity to reduce bacterial activity. In contrast, the bacteriophage *S.So.ph* exhibited much higher efficacy, with an inhibition rate of 96.53%, making it the best option among the three treatments (Table 6). Meanwhile, the combination of mix-phages showed a moderate inhibition rate of 48.10%, suggesting a need for improvement in the formulation or concentration to enhance the effectiveness.

Some significant effects of bacteriophage *S.wph* and *S.So.ph* and their mixture on biofilm formation of *S. marcescens* were detected by A 690 nm and percentage of inhibition and summarized in Figure 7. *S.wph, S.So.ph,* and their mixture significantly inhibited biofilm formation of *S. marcescens* compared to the control (untreated cells). Also, there is no significant differences between *S.So.ph* and the mixture of *S.So.ph*+*S.wph.* The maximum biofilm inhibition was recorded using *S.wph.*

Table 5. **Effect of bacteriophages *S.wph* and *S.So.ph* alone or with Ampicillin on *S. marcescens* growth measured by OD_{590}**

Time/h	Control (*S. marcescens*) OD_{590}	*S.wph* OD_{590}	*S.So.ph* OD_{590}	Ampicillin (µg/ml) OD_{590}	Ampicillin + *S.wph* OD_{590}	Ampicillin + *SSo.ph* OD_{590}
0	0.50 ± 0.03	0.50 ± 0.03	0.50 ± 0.03	0.50 ± 0.03	0.50 ± 0.03	0.50 ± 0.03
2	0.95 ± 0.05	0.30 ± 0.02	0.94 ± 0.05	0.94 ± 0.05	0.30 ± 0.02	0.45 ± 0.03
4	1.40 ± 0.10	0.15 ± 0.01	1.38 ± 0.10	1.38 ± 0.10	0.15 ± 0.01	0.35 ± 0.02
6	1.80 ± 0.15	0.08 ± 0.01	1.78 ± 0.15	1.78 ± 0.15	0.08 ± 0.01	0.25 ± 0.02
8	2.20 ± 0.20	0.05 ± 0.005	2.18 ± 0.20	2.18 ± 0.20	0.05 ± 0.005	0.18 ± 0.01
24	2.60 ± 0.25	0.02 ± 0.003	2.58 ± 0.25	2.58 ± 0.25	0.02 ± 0.003	0.10 ± 0.01
48	2.50 ± 0.20	0.03 ± 0.005	2.48 ± 0.20	2.48 ± 0.20	0.03 ± 0.005	0.12 ± 0.01

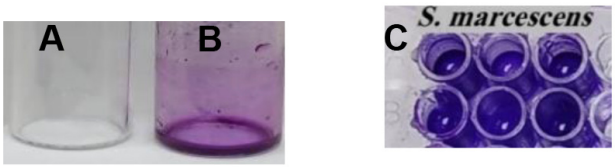

Fig. 5 **The biofilm formation by the tested bacterium using tube method. A: Contains only media and serves as a control. B: Inoculated with *S. marcescens* bacteria C: Stained bacterial biofilm with crystal violet in Microtiter Plate.**

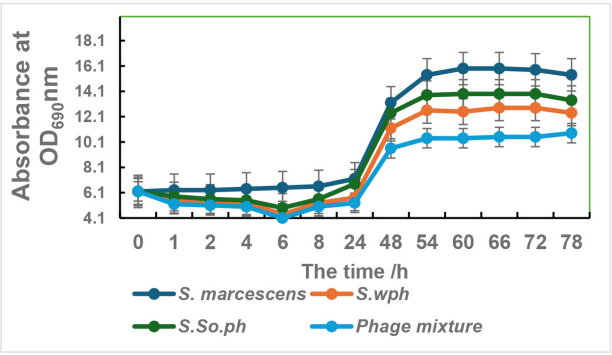

Fig. 6 **Effect of bacteriophage *S.wph* and *S.So.ph* on biofilm formation (A_{690nm}) of *S. marcescens* after different incubation periods.**

Table 6. **Effect of bacteriophages on *S. marcescens* biofilm formation**

Treatment	Biofilm (A_{690nm})	Biofilm inhibition (%)	Degree of biofilm
S. marcescens	2.023	0.0	Strong
S.wph	1.084	53.58	Moderate
S.So.ph	1.953	96.53	Weak
Phage mixture	0.973	48.10	Moderate

EPS was extracted from bacteria treated with *S.wph, S.So.ph,* or their mixture, and the quantity of carbohydrates was calculated from the standard curve of glucose (Figure 8). The effects of phages on the formation of EPS in *S. marcescens* are presented in Table 7, and regarding EPS secretion after treatment with the phages, results showed that the wastewater phage significantly reduced EPS secretion, recording an absorption value of 0.351. In contrast, the soil phage had a lesser effect with an absorption value of 1.219. The mixture of both phages significantly reduced EPS secretion, recording an absorption value of 0.28. Regarding biological activity related to EPS secretion, the wastewater phage recorded 66.66% inhibition compared to 26.57% inhibition for the soil phage. The combination of both phages exhibited an even stronger inhibitory effect on EPS secretion, achieving 80% inhibition. Figure 9 showed the statistical effects of different bacteriophages on EPS production by *S. marcescens* measured by A_{490} nm, the used phage concentration that affects biofilm, and the percentage of EPS inhibition (Figure 9).

Discussion

Bacteriophages specifically infect bacteria and have gained considerable attention in recent years in treating resistant bacteria.[48] The isolation of bacteriophages can be conducted from various environmental sources, including soil and wastewater, where both bacteria and phages coexist. This study focused on the isolation of bacteriophages that target *S. marcescens*, a notable opportunistic pathogen associated with infections in humans and animals[49] and provides a detailed methodology for isolating bacteriophages from environmental sources with potential applications for antimicrobial therapies. Soil and wastewater samples were collected from different locations and used for the isolation of bacteriophages using *S. marcescens* as a host bacterial system. The isolation procedure successfully yielded several phage plaques on agar plates. Preliminary morphological observations of plaques indicated the presence of two bacteriophages which were isolated from soil and wastewater on LB containing *S. marcescens*, and clear zones of lysis (plaques) were counted, and single plaques were picked for further characterization. The results indicated the potential of soil and wastewater as rich sources of bacteriophages specifically targeting *S. marcescens*. The ability of these phages to reduce bacterial populations presents a promising approach to address antibiotic resistance.[50,51]

Historically, physiological studies of phages have often been conducted at concentrations at least 1×10^8 PFU/ml, although achieving these concentrations presents challenges. It can be difficult to obtain such high concentrations of phage particles for some reasons, which lower the discovery, characterization, and application of newly identified phages.[52] The high titers obtained about a bacterial concentration of 1.5×10^8 CFU/ml indicate strong phage replication and lytic

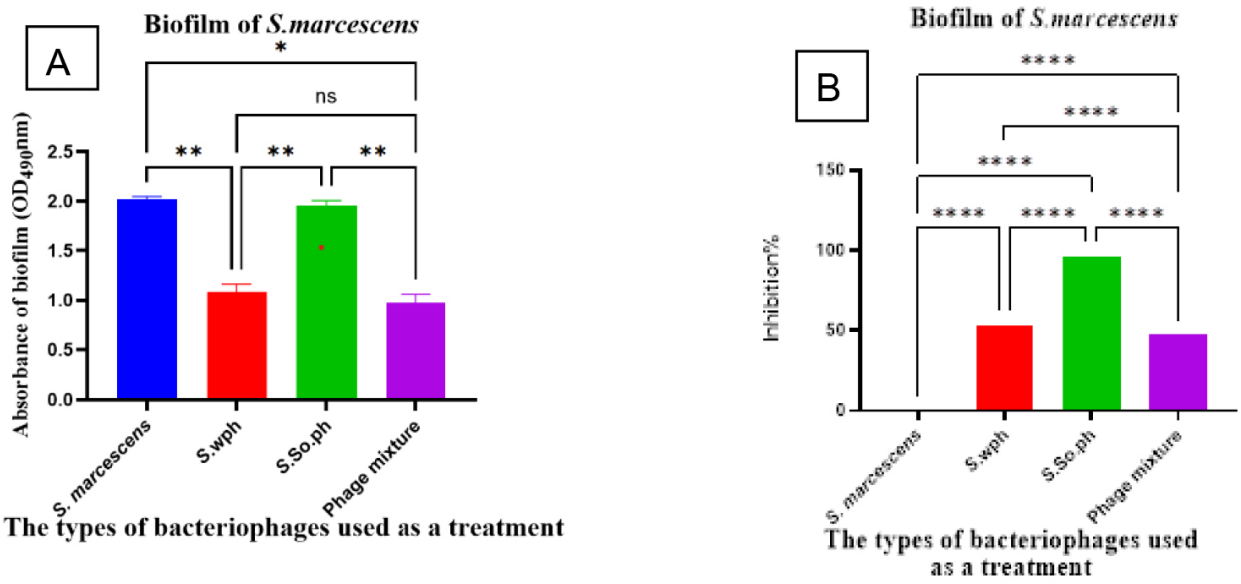

Fig. 7 **Effect of bacteriophage *S.wph* and *S.So.ph* and their mixture on biofilm formation of *S. marcescens* detected by A 690nm (A) and percentage of inhibition (B).**

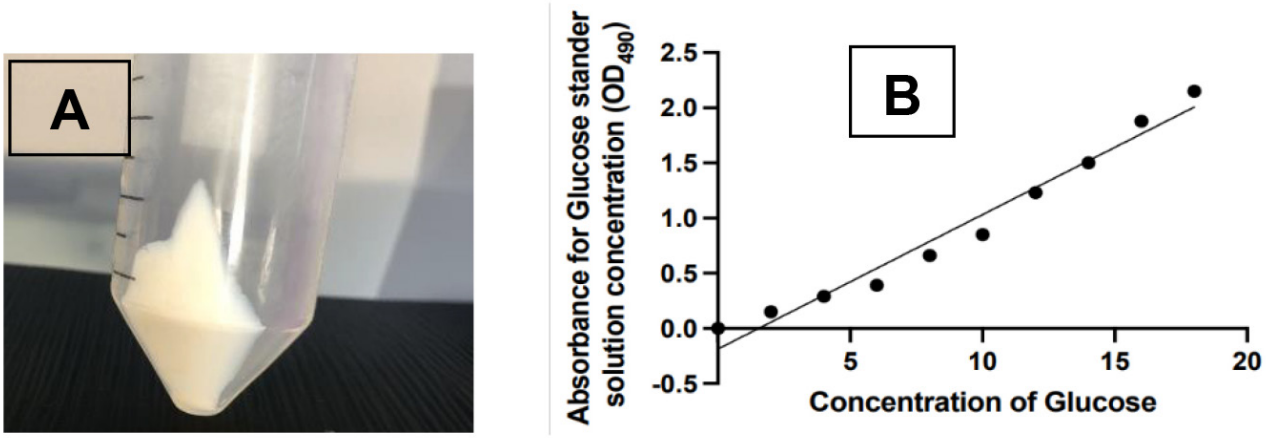

Fig. 8 **A: The extraction exopolysaccharides (EPS) from *Serratia marcescens* and B: Glucose standard curve to detect the content of EPS of the biofilm.**

Table 7. **Impact of bacteriophages on exopolysaccharide (EPS) production by *S. marcescens***

Treatment	EPS (OD_{490} nm)	Activity (%)	Inhibition (%)
S. marcescens (control)	1.75	100	0
S.wph	0.351	33.34	66.66
S.So.ph	1.219	73.43	26.57
Phage mixture	0.28	20	80

activity. The successful isolation of active phages from both wastewater and soil highlights the prevalence of bacteriophages in various environmental niches, as well as their potential to target clinically significant pathogens like *S. marcescens*. The slight difference in phage titers between the two sources suggests that both environments can serve as rich reservoirs for phage isolation, which has important implications for the development of phage therapy and studies in environmental microbiology.

The broader pH and temperature stability of these phages, particularly *S.w.ph*, indicates potential advantages for therapeutic use across diverse environmental conditions. Bacteriophages typically exhibit optimal stability in neutral to slightly alkaline pH ranges, generally around pH 7 to 9. For instance, studies have shown that T4 bacteriophage remains active at pH levels between 5 and 9, with maximum stability observed at neutral pH.[53] In terms of temperature, many bacteriophages can tolerate a wide range, with common stability observed between 4°C and 37°C. Temperate phages like lambda phage show stability at refrigeration temperatures, allowing them to maintain their activity for extended periods.[54] However, extreme temperatures can affect their stability; while some phages can withstand heat treatment, others may lose infectivity above 60°C. The ability of bacteriophages to remain stable under adverse conditions makes them unique candidates for applications in food safety, agriculture, and clinical therapy.

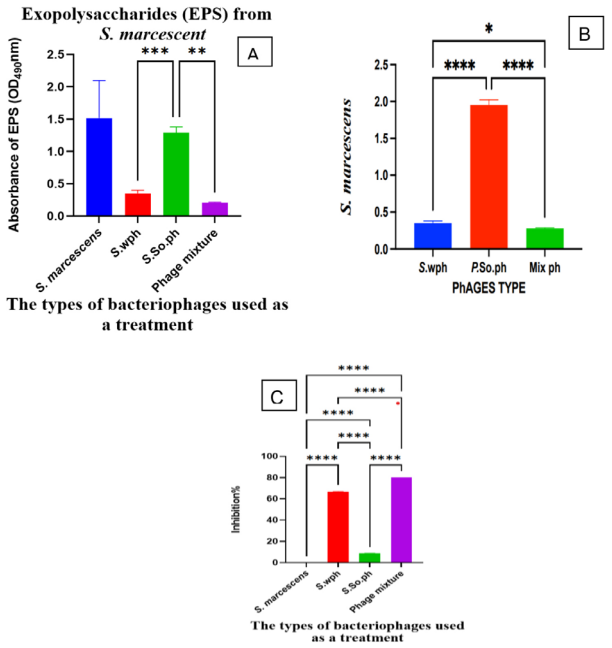

Fig. 9 **The effect of different bacteriophages on exopolysaccharide (EPS) production by *S. marcescens* measured by A490 nm (A), phage concentration that affects biofilm (B), and percentage of inhibition (C).**

The host range of the isolated phages was assessed by performing spot tests on different bacterial strains, including other opportunistic pathogens like *K. pneumoniae* and *E. coli*. The broad-spectrum activity of *S.wph* suggests potential applications in multi-species antimicrobial strategies, while *S.So.ph*'s specificity aligns with typical phage-host interactions. The understanding of host range is crucial for developing phage therapy strategies, as broad-spectrum phages could be more effective in treating infections caused by mixed bacterial populations.[55] The wild host range of bacteriophages can vary significantly among different phage types.[50,51] Many bacteriophages exhibit a narrow host range, meaning they can infect only a specific strain or closely related strains of bacteria. This specificity is a characteristic feature of most traditional phages, like T4 phage, which specifically targets *E. coli*.[56] Conversely, some bacteriophages can infect a broad spectrum of bacterial species, which can be advantageous for therapeutic applications where multiple bacterial targets are present.[57] The bacteriophage SF6 has been reported to infect several strains of *S. aureus* and other related species.[58] The ability of phages to infect various bacteria often depends on specific receptor sites on the bacterial surface and variations in these receptors can lead to differences in host range among phages. Studies have demonstrated that changes in bacterial lipopolysaccharides or teichoic acids can significantly affect phage binding and host specificity.[59] The host range can also be influenced by the isolation source of phages as soil or wastewater, may exhibit distinct host ranges due to the diversity of bacterial populations in those environments.[48]

The MIC of bacteriophages refers to the lowest concentration of phages required to inhibit bacterial growth and is essential for understanding the efficacy of phage therapy, particularly against antibiotic-resistant strains. To calculate the MIC of bacteriophages, standard methods can be employed, similar to those used for antibiotics. Serial dilutions of bacteriophage solutions are prepared, and each dilution is added to a culture of the target bacteria. The cultures are then incubated, and bacterial growth can be assessed either visually or quantitatively (optical density). Rodriguez-Rubio et al. (2014)[60] indicated that the MIC of a specific bacteriophage against antibiotic-resistant *E. coli* was found to be significantly lower than that of traditional antibiotics, suggesting enhanced efficacy of phage therapy. Also, Kwan et al. (2019)[61] reported MIC values for bacteriophages targeting *S. marcescens*, where higher concentrations of bacteriophages resulted in a more substantial reduction of bacterial colonies *in vitro*.

This potent effect corroborates previous findings on the potential of phage therapy in combating antibiotic-resistant bacteria.[62] In this study, ampicillin exhibited minimal inhibitory effects, with bacterial growth patterns closely mirroring untreated control. Notably, the combination of ampicillin with *S.wph* maintained the strong antibacterial effect with no significant differences to *S.wph* monotherapy, suggesting no significant synergistic benefit. However, the ampicillin+ *S.So.ph* combination displayed a moderate bacterial inhibition compared to ampicillin with *S.wph*, and there is a substantial synergistic effect between *S.So.*ph and the antibiotic. This potential synergistic interaction may be considered important in treating resistant bacteria. This observation aligns with recent studies exploring phage-antibiotic synergy as a promising strategy to combat bacterial infections.[63] These findings contribute to the growing body of evidence supporting the efficacy of phage therapy as an alternative or adjunct to conventional antibiotics.[64]

Under normal conditions, the selected *S. marcescens* produced strong biofilm and was resistant to many antibiotics. The visible accumulation of crystal violet-stained biomass on the tube walls proves the bacteria's capacity to form biofilms under the given experimental conditions, and the presence of these adherent, stained structures indicates successful attachment and proliferation of *S. marcescens* cells, as well as the production of EPS. These observations confirm *S. marcescens* ability to transition from planktonic growth to a sessile, biofilm-associated lifestyle under the provided nutrient and environmental conditions and this finding aligns with previous studies documenting the biofilm-forming capabilities of various *Serratia* species and the importance of considering biofilm dynamics in the context of *S. marcescens* infections and persistence.[65]

Bacteriophages are promising options for combating bacterial infections by reducing biofilm formation. Three treatments have been evaluated for their effectiveness against biofilm formation by *S. marcescens,* which were *S.wph*, *S.So.ph*, and a combination of both bacteriophages (*S.wph* and *S.So.ph*). The results showed that the phage isolated from wastewater (*S.wph*) was more effective in reducing biofilm formation. Combination of both phages resulted in a moderate inhibitory effect, achieving 46% inhibition. These findings align with several previous studies demonstrating the effectiveness of bacteriophages in combating bacterial biofilms. For instance, Chegini et al. (2020)[66] reported that bacteriophage PA1Ø showed significant activity against *P. aeruginosa* biofilms and DW-EC exhibited biofilm inhibition capabilities for various *E. coli* strains, with inhibition rates ranging from 39.28–48.13%. Additionally, FP43 reduced *E. coli* O157:H7 biofilm formation by up to 82.4%. Similarly, Chegini et al. (2020)[66] found that using a mixture

of bacteriophages enhanced *P. aeruginosa* biofilm control, achieving a 72.9% reduction in comparison to approximately 50% when individual bacteriophages were used separately. Furthermore, combinations of bacteriophages resulted in maximum *E. coli* biofilm inhibition rates of 86.87%.[67]

Biofilm formation was quantitatively assessed through the measurement of EPS production by the bacterial strains which are key components of biofilms and play a crucial role in protecting bacteria from antimicrobial agents. The quantity of EPS was achieved by determining the total carbohydrate content in the tested isolates via spectrophotometric analysis. The results were subsequently compared to glucose standard solutions for validation. Similarly, Elahi et al. (2021)[68] confirm that phages isolated from wastewater were more effective in reducing biofilm formation and EPS production compared to those isolated from soil. Their results indicated that wastewater-derived phages were capable of inhibiting biofilm formation more effectively, which is consistent with our findings showing greater effectiveness of *S.wph* in reducing both biofilm absorption and biological activity. Similarly, Zhang et al. (2020)[69] demonstrated that wastewater-isolated phages could significantly reduce EPS secretion, thereby decreasing bacteria's ability to form biofilms. This aligns with our results, where *S.wph* showed superior efficacy in reducing EPS secretion compared to *S.So.ph*. On the other hand, Jensen et al. (2020)[70] suggested that mixing phages from different sources could enhance biofilm inhibition compared to using a single phage alone. The combination of soil and wastewater-derived phages resulted in a moderate inhibitory effect on biofilm formation. Additionally, Li et al. (2021)[71] highlighted that soil-isolated phages are generally less effective than those derived from wastewater in inhibiting biofilm growth in *S. marcescens*. Their study emphasized that wastewater-derived phages are more capable of disrupting the biofilm matrix formed by bacteria due to their adaptation to harsher environmental conditions, which is consistent with our findings showing a greater inhibitory effect by *S.wph*. A recent study by Walton et al., (2024)[72] studied the effect of bacteriophage on biofilm of *P. aeruginosa* using the exopolysaccharide as receptor while Wang et al. (2023)[7] added that phages isolated from polluted environments such as wastewater were more effective in reducing bacterial biofilms compared to those isolated from cleaner environments like soil. They also noted that wastewater-derived phages exhibit greater diversity and resilience under challenging conditions, contributing to their enhanced effectiveness in inhibiting biofilms. These phages disrupt biofilm formation, which is important since *S. marcescens* forms resilient biofilms that shield it from antibiotics. Certain phages can reduce EPS production, essential for the biofilm matrix, making bacteria more vulnerable to antibiotics and the immune system. The two studied phages from soil and wastewater showed strong effectiveness in reducing EPS production and disrupting biofilms in antibiotic-resistant *S. marcescens*. Phages are natural enemies of bacteria and can eradicate biofilm formation using several mechanisms as they contain a variety of enzymes, such as depolymerases and lysins that break down the defense barrier during infections of the host bacteria. Pires et al. (2016)[73] recorded 160 putative depolymerases in 143 phages, which can bind and digest EPSs of the host bacterial cells to disturb the biofilm structure, facilitating their penetration to the cells within the biofilm layers.

Conclusion

The natural predators bacteriophages isolated from wastewater were significantly more effective than those isolated from soil in reducing bacterial growth, biofilm formation, and EPS secretion by *S. marcescens* and this could provide an alternative therapeutic strategy against persistent infections caused by *S. marcescens*. The potential to use phage cocktails, combinations of two different bacteriophages or with antibiotics, offers a promising approach to target resistant strains that are difficult to treat with conventional antibiotics and ensure effectiveness in clinical treatments. Such studies support the need for further exploration of phage therapy in clinical settings, particularly against pathogen-driven infections.

Conflict of Interest

None.

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