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Green approach to phytopathogen: Characterization of lytic bacteriophages of *Pseudomonas* sp., an etiology of the bacterial blight of pomegranate

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Authors are dedicating this research article to Late. Prof. Hans-Wolfgang Ackermann for his remarkable contribution to bacteriophage taxonomy and electron microscopy of bacteriophages

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ABSTRACT

Two morphologically different bacteriophages were isolated from the river and soil samples from various locations of Maharashtra, India against the phytopathogen *Pseudomonas* sp. that was recently reported to cause a new bacterial blight of pomegranate. Both the phages belonged to the order *Caudovirales* representing the families *Siphoviridae* (vB_Psp.S_PRϕL2) and *Myoviridae* (vB_Psp.M_SSϕL8). The multiplicity of infection ranged from 0.01 to 0.1, phage adsorption rate from 39% to 66%, latent period from 10 to 20 min with a burst size of 24–85 phage particles per infected host cell. The genome size of phages PRϕL2 and SSϕL8 was approximately 25.403 kb and 29.877 kb respectively. Restriction digestion pattern of phage genomic DNA was carried out for phage PRϕL2, *Eco* RI resulted in two bands and *Hind* III resulted in three bands while for phage SSϕL8, both *Eco* RI and *Hind* III each resulted in three bands. SDS-PAGE protein profile showed six bands for PRϕL2 and nine bands for SSϕL8 of different proteins. Phages showed high pH stability over a range of 4–9, temperature stability over a range of 4–50 °C and UV radiation showed a reduction up to 89.36% for PRϕL2 and 96% for SSϕL8. In short, the present research work discusses for the first time in-detailed characterization of phages of a phytopathogen *Pseudomonas* sp. from Maharashtra, India, which can be further efficiently used for biological control of the causative agent of a new bacterial blight disease of pomegranate.

1. Introduction

Pomegranate scientifically known as *Punica granatum* L. is also called as 'Fruit of Paradise' or 'Super food'. Pomegranate as whole fruit or in the form of processed pomegranate products has enormous economic value. Traditionally, pomegranate has various health benefits due to its nutritional and medicinal properties (da Silva et al., 2013). Pomegranate has a major contribution in several industrial food products such as pomegranate juice, wine, fruit concentrate, beverages, syrup, jelly, pomegranate jam, anardana, etc (Singh and Singh, 2004).

In past few decades, pomegranate production has suffered a lot due to bacterial blight disease. Bacterial blight disease of pomegranate is known to be caused by *Xanthomonas axonopodis* pv. *punicae* (Hingorani and Mehta, 1952; Hingorani and Singh, 1960). Later, the blight disease was reported often throughout India. The production of pomegranate fruit was depleted which resulted in 60–80% reduction of yield thus leading to a great economic loss. Various management strategies failed to overcome the blight disease which mainly included traditional cultural practices, use of antibiotics and agrochemicals (Kumar et al., 2009; Mondal and Mani, 2009) etc. Recently, a new bacterial blight of pomegranate in various regions of Maharashtra, India caused by *Pseudomonas* sp. has been reported (Jagdale et al., 2018). This yellow-pigmented bacterium showed bacterial blight symptoms and the agrochemicals used for the management of this blight along with the traditional agricultural practices were unsuccessful in controlling the disease.

The genus *Pseudomonas* is a Gram negative bacterium categorized as highly pathogenic phytopathogen known to cause a wide variety of plant diseases, which includes blight, spots, streaks, stripes etc. There are numerous pathovars of genus *Pseudomonas* known to cause diseases in economically important plants/crops and which in turn have resulted in massive loss throughout the world (Bradbury, 1986; Höfte and De Vos, 2007). Initially traditional cultural practices were used to control plant infections from spreading from one region to other (Cooksey, 1990; Gašić et al., 2011; Gent and Schwartz, 2005; McManus et al., 2002) but later, the chemical approach was used which included use of antibiotics, copper containing agrochemicals and other pesticides (Cooksey, 1990; McManus et al., 2002). Due to excessive utilization of

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copper containing agrochemicals and antibiotics for the control of plant diseases resulted in not only phytotoxicity but also lead to the development of resistance both chromosomal and plasmid in genus *Pseudomonas*. Therefore, making it more potent and uncontrollable causative agent for various plants (Bender and Cooksey, 1986, 1987; Bender et al., 1990; Höfte and De Vos, 2007). Excess use has lead to an increased level of copper residues in the soil thus resulting in soil pollution (Gent et al., 2005; Köller, 1998).

Under such a situation, it becomes difficult to manage the disease using various methods. Thus, it is necessary to come up with a suitable, target specific, eco-friendly and reliable management strategy i.e. use of bacteriophages as a biocontrol agent. Bacteriophages are host specific thus possess the ability to kill the targeted bacterium (Ackermann, 2007; Mathur et al., 2003). In order to be used as a biocontrol agent, bacteriophages must be lytic i.e. after infecting the target host; phage should undergo lytic cycle thus creating its numerous copies and then release the virulent phages by killing the bacterial host (Sulakvelidze et al., 2001). In addition, as phages are self-limiting in nature they will not cause pollution.

In the present study, we aimed for the isolation and in-detailed characterization of bacteriophages against *Pseudomonas* sp., the etiological agent of new bacterial blight disease of pomegranate in Maharashtra, India. The characterization study will help to understand the efficiency of phages under various environmental conditions and will contribute to the development of phage formulations, which later can be applied in the orchards to overcome the new bacterial blight disease of pomegranate.

2. Materials and methods

2.1. Isolation of pathogen causing bacterial blight of pomegranate

The causative agent of bacterial blight was isolated from infected plant parts of pomegranate from Solapur, Maharashtra, India on sterile Nutrient agar (NA) medium (HiMedia) at 30 °C for 48 h. The alleged colonies of the causal agent was streaked on sterile NA plates as pure culture. The molecular identification was carried out using 16S rRNA sequencing (Salunke et al., 2012). The pure culture of the isolate *Pseudomonas* sp. SK 10 was streaked on sterile NA plates prior to each experiment. In all experiments, for the growth of the bacterial strain in a liquid medium, phage broth (Peptone 10.0 g, Meat Extract 3.0 g, NaCl 5.0 g, Glucose 1.0 g, $CaCl_2.H_2O$ 0.2 g, $MgSO_4$. $7H_2O$ 0.5 g (HiMedia) per litre distilled water and adjusted at pH 7.4) was used. All experiments were standardized with this procedure.

2.2. Isolation, enrichment, and purification of bacteriophages

2.2.1. Sample preparation

The isolation of bacteriophages against pathogen *Pseudomonas* sp. SK 10 was carried from two different environmental samples viz., Pavana river water (Pune, Maharashtra) and pomegranate orchard soil infested with phytopathogen of new bacterial blight disease of pomegranate (Solapur, Maharashtra). The water sample (100 mL) from Pavana river was collected and centrifuged (KUBOTA, Compact High Speed Refrigerated Centrifuge 6500) at 10,000 rpm for 30 min at 4 °C. Similarly, soil sample (10 g) was suspended in 100 mL sterile distilled water (DW), mixed vigorously and then soil was allowed to settle down. The resulting supernatant obtained was collected and centrifuged at 10,000 rpm for 30 min at 4 °C. The respective supernatants obtained were filter sterilized using syringe filter (Cellulose acetate, Axiva, Cat No-SFCA25R) of 0.2 µm pore size to get rid of bacteria present in the samples. Few drops of 10% (v/v) chloroform (S D Fine-Chem Limited) were added to the filtrate and stored at 4 °C.

2.2.2. Isolation of bacteriophages

The presence of bacteriophages in both the filtrates was checked using double agar plaque assay method as described by Adams (1959) with slight modifications. The 100 µL mid-log phase *Pseudomonas* sp. SK 10 culture was mixed separately with 100 µL filtrate obtained from different sources in 5 mL sterile soft agar and overlaid separately on NA plates. After solidification of the soft agar, the plates were incubated at 30 °C for 18 h for development of plaques.

2.2.3. Enrichment of bacteriophages

The phages obtained from Pavana river water and pomegranate orchard soil were named as vB_Psp.S_PRϕL2 and vB_Psp.M_SSϕL8 respectively. Single plaque obtained from both the filtrates were picked up using cork-borer of relevant size and suspended separately in the phage broth containing mid-log phase bacterial culture. The flasks were incubated at 30 °C on rotator shaker (Bio Technics India) at 150 rpm for 24 h. After incubation, the phage broth enriched with respective phage was centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatant from each was filtered through 0.2 µm pore size syringe filters. The resulting lysates of both the phages were stored at 4 °C.

2.2.4. Purification of bacteriophages

In order to raise the probability of obtaining single bacteriophage, purification of the phages PRϕL2 and SSϕL8 was carried out separately using double agar plaque assay method. The mid-log phase bacterial culture was mixed separately with 100 µL lysate of each phage separately in 5 mL sterile soft agar and poured on NA base agar plates. The content of the soft agar was allowed to solidify and plates were incubated at 30 °C for 18 h for development of plaques. The single plaque was again picked up using cork-borer and enriched in phage broth with mid-log phase bacterial culture and incubated at 30 °C on rotator shaker at 150 rpm for 24 h. After 24 h, phage broth of phages PRϕL2 and SSϕL8 were centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatants obtained were filtered using 0.2 µm pore size syringe filters. The lysates thus obtained for both phages were stored at 4 °C. This purification process was repeated for three successive times to ensure purity of the respective phages. The titres of the respective phages as plaque forming units (pfu/mL) were determined.

2.3. Phage morphology

The purified and enriched bacteriophages each with high titre were sedimented at 25,000 × g for 60 min using a Beckman J2-21 centrifuge (Beckman Instruments, Palo Alto, CA). Phages were washed twice in 0.1 M ammonium acetate buffer (pH 7.0) and stained with 2% phosphotungstate (Abcam Biochemicals) (pH 7.2) solution. It is then deposited on the U.V activated carbon-coated Formvar films, air dried and examined under a Philips EM 300 electron microscope (Ackermann, 2005, 2007).

2.4. Host range of bacteriophages

Phages are host-specific in nature. The ability of the phages (PRϕL2 and SSϕL8) to infect different genus of bacteria was determined by spot assay method described by Manchester (1997). The cross infectivity of phages was checked against bacteria of different genera viz., *Xanthomonas*, *Pseudomonas*, *Pantoea*, *Rhizobium*, *Azotobacter* and *Escherichia*. The 100 µL of mid-log phase cultures of respective bacteria was spread on sterile NA plates. These plates were spotted with 10 µL of both phages (PRϕL2 and SSϕL8) respectively. The spots were allowed to air dry and the plates were incubated at 30 °C for 18 h. Clear area of lytic activity against the lawn of bacteria represents its ability to kill the specified bacteria while lack of lytic activity indicated no difference in appearance with that of the surrounding region on the plates.

2.5. Multiplicity of infection

The multiplicity of infection (MOI) is defined as the ratio between

virus particles and host cells. The optimum MOI of both phages (PRϕL2and SSϕL8) was determined as described by Gašić et al. (2011) with slight modifications. *Pseudomonas* sp. SK 10 was grown in phage broth at 30 °C until it reached approximately 10^8 cfu/mL (OD_{600} = 0.250). Bacterial count was checked by counting colony forming units (cfu) obtained by dilution plating on NA medium. The 2 mL of mid-log phase *Pseudomonas* sp. SK 10 was infected with phages PRϕL2 and SSϕL8 separately at four different ratios (approx. 0.01, 0.1, 1, and 10 pfu/cfu) and incubated at 30 °C on a rotary shaker at 150 rpm, for 18 h. After incubation, phage broth of respective MOI was treated with chloroform (10% v/v), centrifuged at 10,000 rpm for 10 min. The supernatant thus obtained was filtered through 0.2 µm pore size syringe filters and the lysates were assayed to determine the phage titre of respective MOI ratio. Assays were performed in triplicate. Of the four different ratios studied, the MOI resulting in highest phage titre within 18 h incubation was considered as an optimum and was used in subsequent phage propagation. The experiment was repeated thrice.

2.6. Phage adsorption

To determine the number of phage particles (PRϕL2 and SSϕL8) adsorbed to host cells within a particular period; an adsorption experiment was carried out as described by Ellis and Delbrück (1939) with slight modification. The 2 mL of mid-log phase *Pseudomonas* sp. SK 10 (10^8 cfu/mL) in phage broth was infected with phage lysate of phages PRϕL2 and SSϕL8 separately to reach MOI of 0.1 and incubated at 30 °C in shaking water bath at 100 rpm. At desired time intervals, (1, 3, 5, 7, 10 and 20 min) aliquots were collected and chloroform treated (10% v/v) in ice cold sodium magnesium (SM) buffer and centrifuged at 10,000 rpm for 1 min to sediment the phage-adsorbed bacteria. The supernatants obtained were filtered with 0.2 µm pore size syringe filters to avoid bacterial cells. Plaque assay was performed to determine the count of unadsorbed phages. The experiment was repeated thrice.

2.7. One step growth curve

The one step growth curve was carried out to determine the latent period and burst size of phages PRϕL2 and SSϕL8 as described by Ellis and Delbrück (1939) with few modifications. The *Pseudomonas* sp. SK 10 was grown in phage broth till it reached titre of 10^8 cfu/mL and then phage lysate of phages PRϕL2 and SSϕL8 were added separately to reach MOI of 0.1 and incubated at 30 °C in shaking water bath (Julabo SW22) at 100 rpm. The phages were allowed to adsorb for 20 min, then the respective phage broth was centrifuged at 10,000 rpm for 5 min to settle the adsorbed phages. The supernatant was discarded and the sediment of the bacteria-phage complex was resuspended in 2 mL phage broth for both phages respectively and was incubated at 30 °C on shaking water bath at 100 rpm. The aliquots (0.1 mL) were collected for respective phages after each 10 min for a period of 100 min. The count of phages was determined by the plaque assay method and the plates were incubated at 30 °C for 18 h.

2.8. Isolation of bacteriophage DNA

For isolation of phage DNA, high titre lysate of phages was prepared using enrichment technique with MOI of 0.1 for phages PRϕL2 and SSϕL8 respectively. The concentration of respective phage was carried out by using caesium chloride density gradient centrifugation as mentioned by Davis et al. (1980) employing 24,000 rpm for 2 h at 4 °C using centrifuge (BioEra Life Sciences Pvt. Ltd., Pune, India). Extraction of phage DNA was carried out as per method given by Ausubel et al. (1999) with slight modifications and was purified. The size of the purified phage DNA was determined using 0.5% agarose gel electrophoresis along with a standard molecular weight marker and later agarose gel was then examined using Alpha Image Software in BioEra Gel Documentation System (BioEra Life Sciences Pvt. Ltd., Pune, India).

2.9. Restriction digestion analysis

The purified phage DNA was subjected to two different restriction endonucleases to obtain restriction profile. An aliquot (20 µL) of each phage DNA was digested with 1 µL of *Eco* R1 and *Hind* III separately with 2.5 µL of 10X assay buffer along with 1.5 µL nuclease free water. The reaction was carried out in a water bath at 37 °C for 1 h. Then 5 µL of 5X DNA loading dye was added to the mixture to terminate the reaction. The 0.5% agarose for submarine gel electrophoresis was used to examine the product formed with DNA molecular weight marker having a broad range. The agarose gel was then examined using Alpha Image Software in BioEra Gel Documentation System.

2.10. Protein profiling

The high titre phage lysates were obtained for protein analysis as described above. For extraction of proteins, first the phage lysates were concentrated using 10% polyethylene glycol (PEG) 8000 (HiMedia) and then incubated overnight at 4 °C. Further, after incubation the precipitate thus obtained was subjected to centrifugation at 12,000 × g for 25 min to obtain a glazy pellet. The supernatant was discarded while SM buffer was added to the glazy pellet to resuspend the respective pellets of both phages. The phages were further concentrated with the help of Amicon (Millipore India Pvt. Ltd, Bangalore, India) assembly with 3 kDa cutoff membrane. Disruption of phage particles was carried out with the help of Triple Freeze Thawing Cycle as described by Lavigne et al. (2006). The phage protein obtained was then analyzed by loading on 12% SDS-PAGE using Vertical Electrophoresis System and power supply (BioEra Life Sciences Pvt. Ltd., Pune, India) with the standard broad range protein molecular weight marker (BioEra Life Sciences Pvt. Ltd., Pune, India) as illustrated by Laemmli (1970). Coomassie dye G-250 (Sigma-Aldrich, Bangalore, India) was used for visualization of protein bands obtained for both phages.

2.11. RAPD-PCR analysis of phage DNA

The purified phage DNA was subjected to random amplified polymorphic DNA analysis as described by Gutiérrez et al. (2011). In short, Primers OPL5 (5′- ACGCAGGCAC-3′), RAPD5 (5′-AACGCGCAAC-3′), P1 (5′-CCGCAGCCAA-3′) and P2 (5′-AACGGGCAGA-3′) were used at 8 u M concentration for the RAPD-PCR analysis. 10–15 ng of purified phage DNA was mixed with Dream Taq PCR master mix (Fermentas) to perform the PCR reaction in a thermal cycler (BioEra, Gradient PCR Processor). The amplification was allowed by following the PCR conditions as mentioned by Gutiérrez et al. (2011). PCR product were resolved on 1.5% agarose gel and stained with ethidium bromide. The product formed was examined with DNA molecular weight marker having a broad range along with phage genomic DNA. The agarose gel was then examined using Alpha Image Software in BioEra Gel Documentation System.

2.12. Effect of different environmental factors

Bacteriophages have potential to be used as a biocontrol agent against plant diseases caused by bacteria. In the orchards, the phyllosphere conditions are critical, so being proteinic in nature phages will degenerate quickly (Jones et al., 2007). In order to be utilized as a biocontrol agent, more stable and efficient phages should be selected. For this purpose, phages were characterized for their stability over a wide range of pH and temperature. In addition, the stability of phages was checked under direct UV source (Czajkowski et al., 2014) to determine its ability to sustain the harsh sunlight in the field, thus they can be used in field applications as a biocontrol agent.

2.12.1. Effect of pH on stability of bacteriophages

The effect of pH on phage stability was studied for respective phages

with pH values (pH 1–14) in sterile SM buffer at 30 °C as described by Czajkowski et al. (2014) with slight modifications. The phage lysate of PRϕL2 and SSϕL8 respectively were added to sterile SM buffer adjusted to different pH values. These tubes were incubated at 30 °C for 1 h. After incubation, a plaque assay was performed to determine the phage titre as explained above. The experiment was repeated thrice.

2.12.2. Effect of temperature on stability of bacteriophages

The effect of temperature on phage stability was studied at –20, 4, 30, 37, 42, 50, 60, 70, and 80 °C in sterile SM buffer as described by Czajkowski et al. (2014) with slight modifications. The phage lysate of PRϕL2 and SSϕL8 respectively were added to sterile SM buffer adjusted to pH 7.5. These tubes were incubated at different temperatures for 1 h. After incubation, a plaque assay was performed to determine the phage titre as explained above. The experiment was repeated thrice.

2.12.3. Effect of UV radiation on stability of bacteriophages

The effect of UV radiation on phage stability was studied in sterile SM buffer as described by Czajkowski et al. (2014) with slight modifications. The phage lysate of PRϕL2 and SSϕL8 was added to sterile SM buffer placed in the base of a petri plate. Phage lysates were then exposed to UV light (Philips TUV G30T8, UV dose 50 mJ/cm^2 30 cm from the source of UV light) for 5, 10, 15, 30 and 60 min separately for both phages. After the desired interval of time, aliquots of phage lysate of respective phages were removed and plaque assay was performed to determine the phage titre. The experiment was repeated thrice.

3. Results

3.1. Isolation of pathogen causing bacterial blight of pomegranate

The yellow coloured glistening mucoid colonies were obtained on sterile NA plates. The molecular identification using 16S rRNA sequencing revealed that the causal agent was identified as *Pseudomonas* sp. The 16S rRNA sequence of *Pseudomonas* sp. SK 10 was deposited in GeneBank with accession number KY427436.

3.2. Isolation and purification of bacteriophages

From two different environmental samples collected viz., Pavana river water (Pune, Maharashtra) and soil from pomegranate orchards infested with bacterial blight disease (Solapur, Maharashtra), phages were isolated from each sample. Between the two different environmental samples collected, five phages were isolated. Some phages were pinpoint in nature while two showed 1–2 mm range of plaque size and two phages one from each sample, which showed 2 mm diameter plaque size, were selected for further study. All phages were lytic in nature but differed in the plaque diameter. Some showed pinpoint nature while others were greater than that. Out of these phages, PRϕL2 and SSϕL8 were selected for further study as represented in Table 1. Single plaque obtained from each sample was enriched and purified for further studies.

3.3. Phage morphology

One of the major criteria for the identification of phages is its morphological appearance, preferably determined with the aid of Transmission electron microscopy (TEM). The TEM images showed that both phages belonged to order *Caudovirales*. Between the two phages, PRϕL2 phage belonged to family *Siphoviridae* with an isometric head of 72 nm and an extremely long, more of the less flexible tail of about 383 x 12 nm, conspicuous cross striations, and a terminal knob. While phage SSϕL8 belonged to *Myoviridae* family showed an isometric head of 59 nm and a contractile tail of 101 x 17 nm in the extended state. The contractile tail measured 42 x 22 nm in the contracted state. The phage showed a neck, a base plate of 7 x 2 nm and fibres of about 12 x 3 nm with a terminal swelling (Fig. 1).

3.4. Host range of bacteriophages

The phages were checked for their ability to cross infect bacteria of different genus by spot assay method. The bacterial strains viz., *Xanthomonas campestris* MTCC 6843, *Xanthomonas malvacearum* NCIM 2310, *Pseudomonas syringae* MTCC 673, *Pseudomonas fluorescens* MTCC 671, *Pseudomonas aeruginosa* MTCC 424, *Pantoea agglomerans* MTCC 6536, *Azotobacter vinelandii* NCIM 2821, *Rhizobium japonicum* NCIM 2741, and *Escherichia coli* MTCC 1678 were checked for lytic activity by both phages. Phages PRϕL2 and SSϕL8 were unable to lyse *Pseudomonas syringae* MTCC 673, *Pseudomonas fluorescens* MTCC 671, and *Pseudomonas aeruginosa* MTCC 424. In addition, neither of the phages showed lytic activity against other bacterial genera tested in the present study (Table 2).

3.5. Multiplicity of infection, phage adsorption and one step growth curve

The multiplicity of infection (MOI) of both phages was determined separately at four different ratios (approx. 0.01, 0.1, 1, and 10 pfu/cfu). PRϕL2 phage showed optimum MOI at 0.1 while 0.01 was the optimum MOI for phage SSϕL8. The adsorption study was carried out in phage broth (pH 7.4) at 30 °C for 20 min. The % adsorption of PRϕL2 phage was below 30% upto 7 min which rapidly increased to 66% by 20 min. Similarly, % adsorption of SSϕL8 phage was below 30% upto 7 min which increased only slightly to 39% by 20 min (Fig. 2). Thus, the rate of adsorption of PRϕL2 was faster in comparison to SSϕL8. One step growth curve study was performed in phage broth to which phages were added separately to reach MOI of 0.1 and incubated at 30 °C on shaking water bath at 100 rpm for a period of 60 min. Phage growth parameters which included latent period and burst size were determined for both phages. PRϕL2 showed a latent period of 20 min with a burst size of 85 (± 4) phage particles per infected host cell while SSϕL8 revealed 10–20 min of the latent period with a burst size of 24 (± 5) phage particles per infected host cell (Fig. 3).

3.6. DNA isolation (genome size) and restriction digestion analysis

The genomic DNA of phages was isolated and the genome size was determined with the aid of the standard molecular weight marker. The genome size of PRϕL2 and SSϕL8 was 25.403 kb and 29.877 kb respectively (Supplementary Fig. S1). Phage genomic DNA was subjected to restriction digestion with two restriction endonucleases *Eco* RI and *Hind* III respectively. The digestion of genomic DNA of PRϕL2 resulted in two different bands from *Eco* RI and three different bands from *Hind* III. Similarly, digestion of genomic DNA of SSϕL8 resulted three different bands for both the respective restriction enzymes (Fig. 4). The restriction patterns thus obtained distinctly specify that the phages are genetically different from each other.

Table 1
Phages isolated against *Pseudomonas* sp. SK 10 from different sources.

Phages	Source	Nature of plaque	Diameter of plaque (mm)
vB_Psp.S_PRϕL2	Pavana river, Pimpri, Pune	Lytic, circular	2
vB_Psp.M_SSϕL8	Solapur Soil (blight infested pomegranate orchard), Solapur	Lytic, circular	2

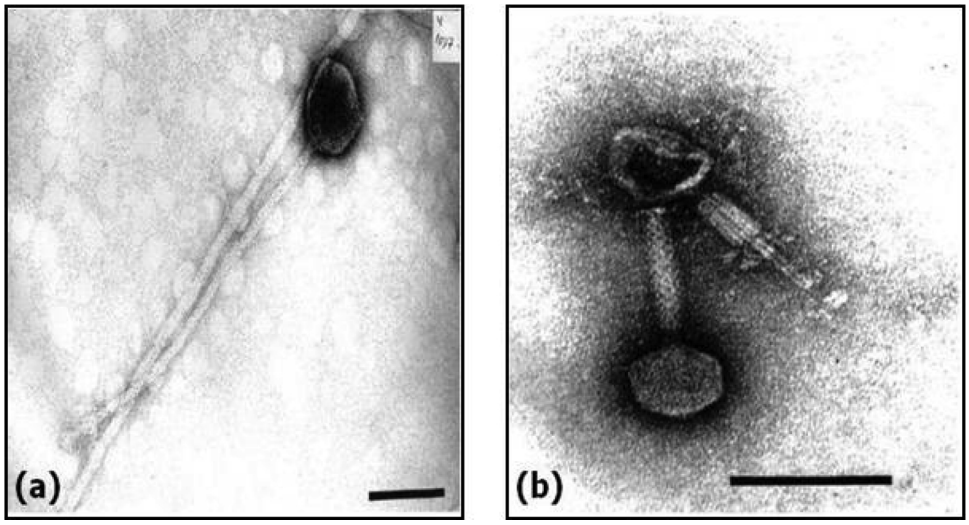

Fig. 1. Transmission electron micrographs of lytic phages (a) vB_Psp.S_PRϕL2 and (b) vB_Psp.M_SSϕL8 of *Pseudomonas* sp. SK 10 stained with 2% phosphotungstate (pH 7.2) solution. The bar indicates 100 nm. Each micrograph illustrates bacteriophage morphology.

Table 2
Host range of isolated phages against different genera of bacteria.

Name of Microorganisms	Lytic activity of PRϕL2[a]	Lytic activity of SSϕL8[a]
Xanthomonas campestris MTCC 6843	–	–
Xanthomonas malvacearum NCIM 2310	–	–
Pseudomonas syringae MTCC 673	–	–
Pseudomonas fluorescens MTCC 671	–	–
Pseudomonas aeruginosa MTCC 424	–	–
Pantoea agglomerans MTCC 6536	–	–
Azotobacter vinelandii NCIM 2821	–	–
Rhizobium japonicum NCIM 2741	–	–
Escherichia coli MTCC 1678	–	–

[a] = '+': lytic activity; and '−': no lytic activity.

3.7. Protein profiling

Phage protein mainly is a combination of different proteins viz., capsid, tail, tail fibres, base plate, and tail pins. Phage proteins were extracted and analyzed using SDS-PAGE and were determined with the aid of standard broad range protein molecular weight marker. Protein bands thus obtained for both phages were visualized using Coomassie dye G-250. PRϕL2 showed the presence of six bands with the band molecular size of 174 kDa being the largest one when distinctly separated on SDS-PAGE while SSϕL8 revealed the presence of nine bands with 191 kDa as the biggest protein (Fig. 5). Thus, protein profiling supports the TEM image of phages where SSϕL8 showed a typical phage structure having all components of phage protein while PRϕL2 lacks some of them.

3.8. RAPD-PCR analysis of phage DNA

Phage genomic DNA was subjected to four different RAPD-PCR primers respectively. It seems that both the phage samples were unable to show specific or prominent bands for all four primers used in the experiment (Supplementary Figs. S2 and S3). The fingerprinting analysis was carried out on three separate days to check reproducibility but unable to form bands on every occasion.

3.9. Effect of different environmental factors

3.9.1. Effect of pH on stability of bacteriophages

The effect of different pH values on phage stability was checked in sterile SM buffer at 30 °C. Both the phages showed optimum stability at

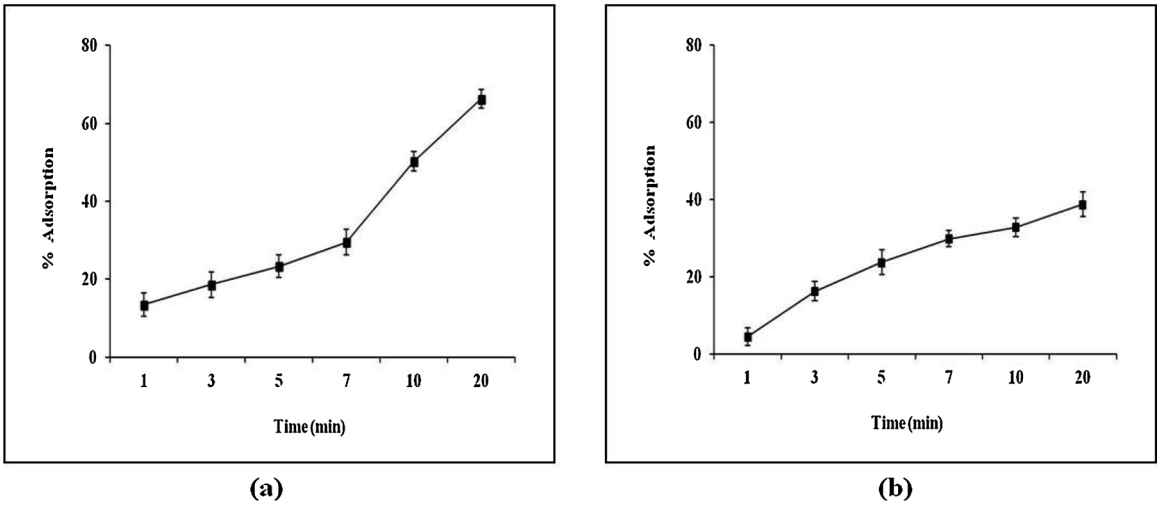

Fig. 2. % Adsorption of phages (a) PRϕL2 and (b) SSϕL8 for *Pseudomonas* sp. SK 10. The experiment was performed to determine the number of phage particles adsorbed to host cells within a particular period. Data are averages of three determinants ± SD.

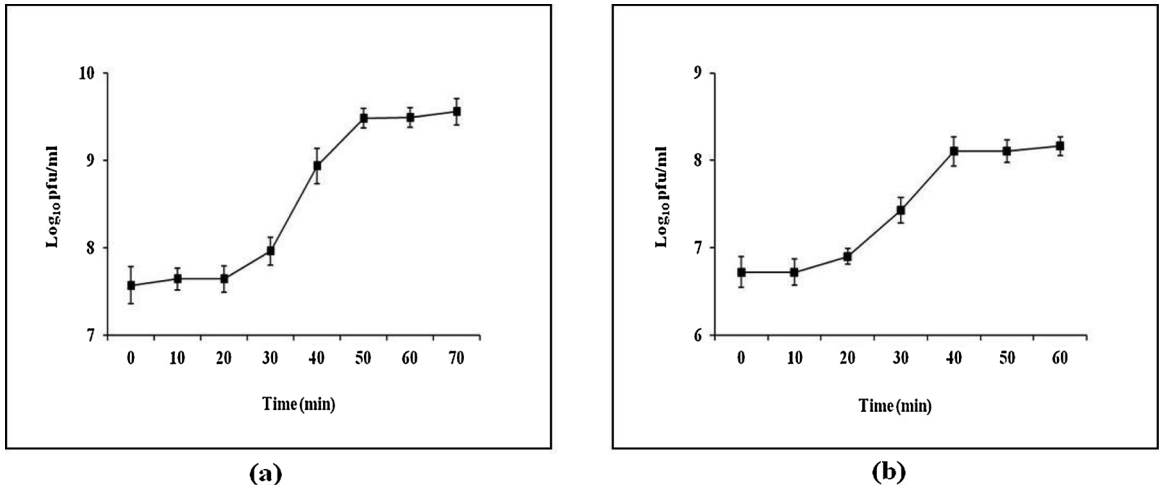

Fig. 3. One step growth curve of phages (a) PRϕL2 and (b) SSϕL8 for *Pseudomonas* sp. SK 10. The experiment was performed to determine the latent period and burst size of phages. Data are averages of three determinants ± SD.

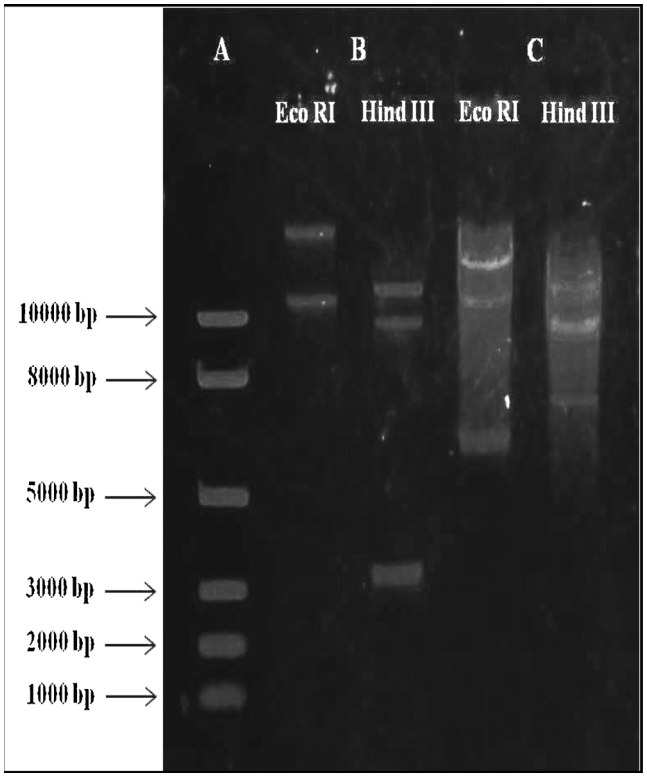

Fig. 4. Restriction digestion pattern obtained of phage DNA (a) PRϕL2 and (b) SSϕL8 after reaction with different restriction enzymes. The resolution of bands is obtained on 0.5% agarose. Lane A, Marker DNA ladder as standard; Lane B-*Eco* R1, *Eco* R1 digested DNA of PRϕL2, and Lane B-*Hind* III, *Hind* III digested DNA of PRϕL2; Lane C-*Eco* R1, *Eco* R1 digested DNA of SSϕL8, and Lane B-*Hind* III, *Hind* III digested DNA of SSϕL8.

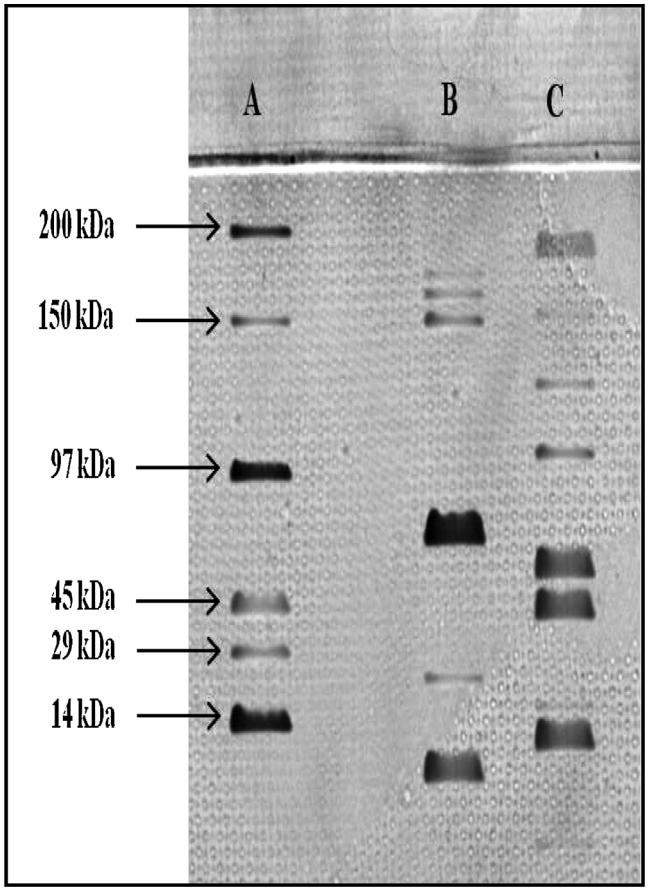

Fig. 5. Protein profiling of phages (a) PRϕL2 and (b) SSϕL8 were analyzed and resolved on 12% SDS-PAGE. Lane A, Marker protein ladder as standard; Lane B, protein bands of phage PRϕL2; and Lane C, protein bands of phage SSϕL8.

pH 7. PRϕL2 showed high stability over pH range of 6–9 with stability above 80% while stability was 77.07% and 70.13% at pH 5 and 4 respectively and 72.25% and 63.97% at pH 10 and 11 respectively. SSϕL8 showed high stability over pH range 4–8 above 80% while stability was 75.58% at pH 3 and stability over pH range of 9–12 was between 79.9-68.96% (Fig. 6). This result indicates that SSϕL8 is more stable over the pH 3–12 than PRϕL2.

3.9.2. Effect of temperature on stability of bacteriophages

The effect of different temperatures on phage stability was checked in sterile SM buffer adjusted at pH 7.5. The phage PRϕL2 showed stability over a wide temperature range from 4 to 50 °C showing 98.32% stability at 28 °C. Stability of PRϕL2 was 87% at 50 °C while it still showed stability up to 64% at 60 °C while − 20 °C stability decreased up to 37%. Similarly, phage SSϕL8 showed stability over wide temperature range same as PRϕL2 from 4 to 50 °C showing 99.9% stability at 28 °C. Stability of SSϕL8 was 95% up to 42 °C while it showed the stability of 60% at 60 °C while the stability decreased rapidly to 1.5% at − 20 °C

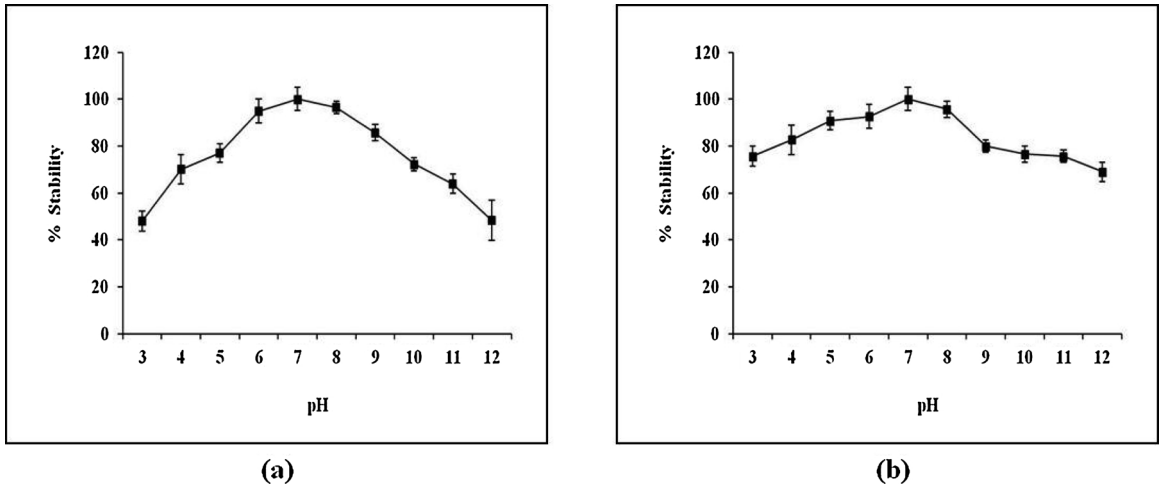

Fig. 6. Effect of different pH on stability of phages (a) PRϕL2 and (b) SSϕL8.% Stability: $N/N_0 \times 100$, where N is the number of viable phage particles after incubation at 30 °C for 1 h at different pH values, N_0 is the number of phages at pH 7; % stability is with reference to phage titre (pfu/mL) at pH 7. Data are averages of three determinants ± SD.

(Fig. 7).

3.9.3. Effect of UV radiation on stability of bacteriophages

The effect of UV radiation on phage stability was studied in sterile SM buffer. Phage PRϕL2 showed 89.36% reduction in phage titre when exposed to direct UV radiation for 30 min. Similarly, phage SSϕL8 showed 96% reduction in phage titre after 30 min of exposure under direct UV radiation (Fig. 8).

4. Discussion

It is universal that phages are ubiquitous in nature and show their existence wherever their hosts are found (Ackermann, 2007; Mathur et al., 2003). For the present study, phages isolated were morphologically distinct which belonged to the families *Siphoviridae* (PRϕL2) and *Myoviridae* (SSϕL8) of the order *Caudovirales* as per classification put forward by Ackermann (2005). Among the phages reported for the genus *Pseudomonas* phytopathogen belonged to order *Caudovirales* as obtained in the present study. Majority of phages isolated against *Pseudomonas syringae* pv. *actinidiae*, that causes bacterial canker in kiwifruit, belonged to family *Myoviridae* while only one phage represented *Siphoviridae* family (Frampton et al., 2014). Similarly, Di Lallo et al. (2014) isolated two phages ϕPSA1 (siphovirus) and ϕPSA2 (podovirus) against *Pseudomonas syringae* pv. *actinidiae*, the causal agent of bacterial canker in kiwifruit. Rombouts et al. (2016). reported the isolated five phages were myovirus against *Pseudomonas syringae* pv. *porri* known to cause bacterial blight in leek. The phage ϕIBB-PF7A isolated against *Pseudomonas fluorescens* belonged to *Podoviridae* family as reported by Sillankorva et al. (2008). Sajben-Nagy et al. (2012) also reported podovirus against *Pseudomonas tolaasii* from the necrotic regions of sporocarps of oyster mushrooms. With an exception, phage of family *Cystoviridae* was isolated against *Pseudomonas putida* that causes a soft rot of potato tubers (Delfan et al., 2012). Phages of *Myoviridae* family have emerged to be the most dominant against phytopathogen *Pseudomonas*. However, there are no reports of phages isolated against *Pseudomonas* sp. SK10 that causes new bacterial blight of pomegranate in Maharashtra, India. Phages PRϕL2 and SSϕL8 showed no lytic activity against the different genera tested in the present study including three different *Pseudomonas* species.

Both the phages isolated from different sources where characterized fully which included MOI, adsorption rate and one step growth curve against the host *Pseudomonas* sp. SK 10. Phages differ not only in their MOI but also in their rate at which they adsorbed on the host, where initially both the phages showed adsorption of 30% till 7 min. Phage

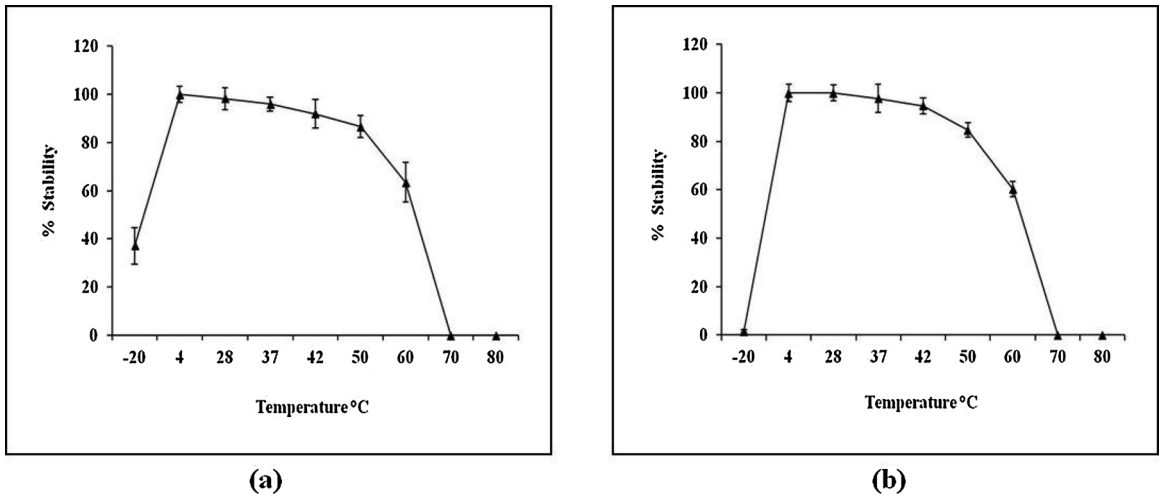

Fig. 7. Effect of different temperatures on the stability of phages (a) PRϕL2 and (b) SSϕL8.% Stability: $N/N_0 \times 100$, where N is the number of viable phage particles after incubation at pH 7.5 for 1 h at different temperature values, N_0 is the number of phages at temperature 4 °C; % stability is with reference to phage titre (pfu/mL) at 4 °C. Data are averages of three determinants ± SD.

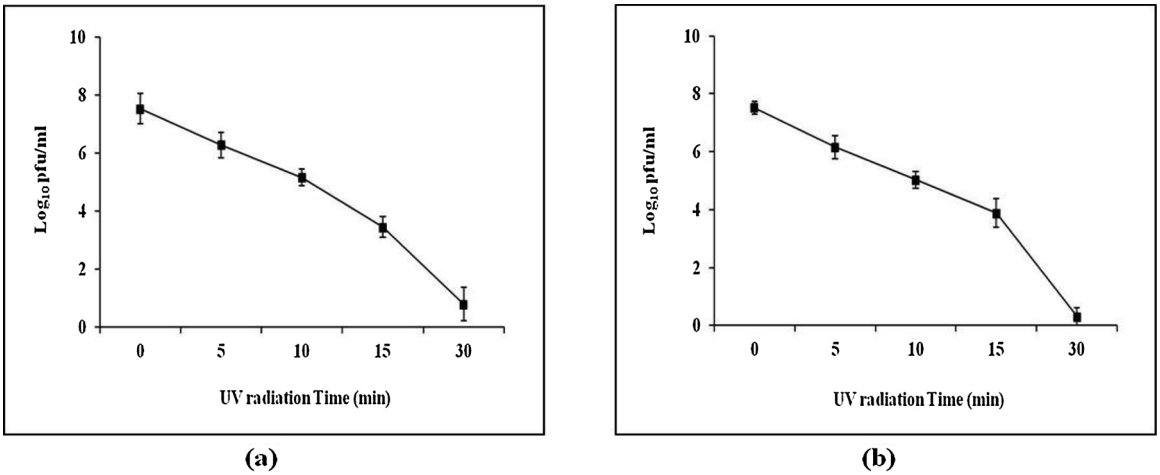

Fig. 8. Effect of UV radiation on the stability of phages (a) PRϕL2 and (b) SSϕL8. The experiment was performed to determine the reduction of phage titre on exposure to UV radiation for the period of 30 min. Data are averages of three determinants ± SD.

PRϕL2 adsorbed on the host at a faster rate of 66% while SSϕL8 was slowly adsorbed with a rate of 39% in a time period of 20 min. The rate of adsorption of both the phages ϕPSA1 and ϕPSA2 isolated against *Pseudomonas syringae* pv. *actinidiae*, the causal agent of bacterial canker in kiwifruit was 30% till 5 min and displayed sluggish adsorption rate up to 40–50% at the end of 30 min (Di Lallo et al., 2014). In the present study, the adsorption rate of both phages is less than that reported by Rombouts et al. (2016) as 99% for phage against *Pseudomonas syringae* pv. *porri*. The latent period and burst size of phages were found to be 20 min and 85 (± 4) phage particles per infected host cell (burst size) for PRϕL2 and 10–20 min and 24 (± 5) phage particles per infected host cell for SSϕL8 respectively. The phage ϕIBB-PF7A isolated against *Pseudomonas fluorescens* showed the latent period of 15 min and 153 phage particles per infected host cell. The burst size of phage ϕIBB-PF7A was more as compared to the burst size of the phages isolated in the present study (Sillankorva et al., 2008). Interestingly, the phage ϕPSA1 isolated against *Pseudomonas syringae* pv. *actinidiae* showed a latent period of 100 min and a burst size of 178 ± 23 while phage ϕPSA2 showed a smaller latent period of 15 min and burst size of 92 ± 21 (Di Lallo et al., 2014). This illustrates that phages isolated from different sources differ in morphology, MOI, adsorption rate, and burst size.

Another major feature in the characterization of phages is the genome size. The phages PRϕL2 and SSϕL8 showed smaller genome size of 25.403 kb and 29.877 kb respectively when compared to the genome size reported against phytopathogen *Pseudomonas*. There are few reports on phages against phytopathogen *Pseudomonas* species with such small genome size. Phages with a genome size of 24 to 49 kb isolated from sewage and 39 to 52.5 kb from culture supernatant reported by Nordeen et al. (1983) against *Pseudomonas syringae* pv. *syringae* mainly were siphoviruses that are comparable to PRϕL2. The genome size of phages obtained against *Pseudomonas syringae* pv. *tomato* which represented the family *Siphoviridae* was in the range of 40–52 kb (Prior et al., 2007) still larger than PRϕL2. There is also a report of siphovirus with a genome size of 110 kb (Frampton et al., 2014) and ϕPSA1 showed genome size of 51.09 kb (Di Lallo et al., 2014) against *Pseudomonas syringae* pv. *actinidiae* which is far more greater than PRϕL2 having a genome size of 25.403 kb. The myoviruses that were reported earlier showed larger genome size of 95–97.8 kb against *Pseudomonas syringae* pv. *actinidiae* (Frampton et al., 2014) and 90–94 kb against *Pseudomonas syringae* pv. *porri* (Rombouts et al., 2016) in comparison to the small genome size of myovirus SSϕL8 isolated in the present study. In addition, the podovirus ϕIBB-PF7A reported against *Pseudomonas fluorescens* showed the genome size of 42 kb (Sillankorva et al., 2008). Similarly, podovirus ϕPSA2 was reported to have a genome size of 40.472 kb (Di Lallo et al., 2014). The complete genome sequencing of the phages would be essential to unveil the information to exactly what extent the phages are related to those, which are reported earlier.

Restriction digestion pattern of phage genomic DNA with *Eco* RI and *Hind* III revealed a different pattern of bands each with different molecular weights. The phages PRϕL2 and SSϕL8 showed different restriction pattern from those which were reported against phages of *Pseudomonas syringae* pv. *syringae* (Nordeen et al., 1983) and phages of *Pseudomonas syringae* pv. *tomato* (Prior et al., 2007) respectively. Therefore, it supports that phages are not only genetically different from one another but also from the one that have been reported. In addition, the SDS-PAGE profiling of phages resulted in six bands for PRϕL2 and nine bands for SSϕL8 of different proteins respectively that supported the TEM images of respective phages in the present study.

The RAPD-PCR of the phages PRϕL2 and SSϕL8 showed no amplification of the primers that are commonly used for identification (Gutiérrez et al., 2011; Roach et al., 2015; Samoilova and Leclerque, 2014). Ellsworth et al. (1993), described primer designing can be performed independent of sequence information. However, the analysis is also dependent on various other factors such as template DNA concentration, PCR conditions and electrophoresis setting. In addition, Pérez et al. (1998) reported that reproducible band formation would also be challenging as the primers non-specifically bind to the template phage DNA. In addition, bacteriophages corresponding to different families may even show same DNA sequence (Ackermann, 2003). Hence, similar RAPD-PCR bands would be possible even if the phages morphologically belong to different families (Merabishvili et al., 2007). Bacteriophage classification into different families thus has mainly been performed depending on the phage morphology and the nucleic acid present (Ackermann, 2003).

Further, phages were also characterized for their ability to sustain harsh environmental conditions, which included a range of pH, temperature, and exposure to direct UV radiations prior to their usage in agriculture fields as biocontrol agents. Phages in the present study showed stability over a wide range of pH for PRϕL2 in between the range of 6–9 while SSϕL8 was found to be stable in the range of 4–8 when incubated at respective pH for a period of 1 h. This was comparable to both the phages ϕPSA1 and ϕPSA2 which showed pH stability from 5 to 9 (Di Lallo et al., 2014). In case of temperature stability, both phages PRϕL2 and SSϕL8 were stable over a broad temperature range from 4 to 50 °C as compared to the phages reported earlier. Phages isolated against *Pseudomonas syringae* pv. *actinidiae* showed stability over a temperature range of 4–25 °C (Frampton et al., 2014) and phages ϕPSA1 and ϕPSA2 showed temperature stability at 40–50 °C

(Di Lallo et al., 2014). Phages of *Pseudomonas syringae* pv. *porri* were stable over a temperature range of 4–37 °C (Rombouts et al., 2016) which were lower than the temperature range showed by the phages studied in the present research work. Phages in the present study when were exposed to direct UV radiation for 30 min showed a reduction in the titre to 89.36% by PRϕL2 and 96% by SSϕL8. The data suggests that phages were able to withstand the UV radiation for far more time than that reported against phages of *Dickeya* spp. where phages showed 50% reduction just after 5 min while none outlived 10 min of exposure (Czajkowski et al., 2014). Phages when exposed to various environmental factors normally tend to decrease their titre, which in turn affects its efficiency when used in field applications (Czajkowski et al., 2014; Jones et al., 2007). The phages in the present study exhibit their stability to various environmental factors thus will potentially aid in controlling the bacterial blight disease in the field.

5. Conclusion

The present work reports detail characterization of bacteriophages against *Pseudomonas* sp. SK10, an etiological agent of the bacterial blight disease of pomegranate from Maharashtra, India. The phages isolated from different regions are morphologically and genetically different and do not kill the other genera of bacteria. Phages showed a wide range of stability under various environmental factors that are mainly encountered in the orchards thus making them potent tool for restraining the causative agent of the blight disease in pomegranate.

Declaration of Competing Interest

There is no conflict of interest among the authors.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.126300.

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