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Isolation and Characterization of Bacteriophage Against *Xanthomonas* Sps. And Their use in Phage Therapy

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Abstract
Diseases of agricultural crops are key risk factors for the farmers in India and whole world. Plant pathogens are responsible for major economic losses in agriculture. Bacterial diseases are generally problematic to be controlled due to lack of effective bactericides and specifically acting chemicals. Resistance developed from pathogen itself is also a major problem in control of disease. An attractive option is the biocontrol using specific bacteriophages (phages), viruses that specifically kill bacteria, providing more targeted approach. Most of the species of *Xanthomonas* are plant pathogen that causes canker disease in lemon fruit, orange, sweet orange, grapes etc. *Xanthomonas* causes high yield loss. *Xanthomonas* sps. isolated from the infected lemon fruit on Starr's agar and Nutrient agar medium and it showed re-infection to lemon fruit. Soil Sample was collected from lemon farm situated at Bhawaninagar, Indapur, Pune of Maharashtra state for isolation of phages. 220 plaque forming unit (PFU) were found per ml of suspension. Formulation was done for increased residual activity of bacteriophages. Skim milk and sucrose was used for formulation of bacteriophage. Application of formulated phage on detached leaves was done (Xan:Phage 2:1). Sucrose formulation shows better control for *Xanthomonas* infection which was detected by quantifying total chlorophyll pigment in normal leaves (11.68 mg/gm) and sucrose formulated leaves (7.80 mg/gm). In this investigation, bacteriophage was isolated and their antimicrobial potential in the management of *Xanthomonas* infection of lemon was evaluated.

Keywords
Xanthomonas, Phage therapy, Bacteriophage, Lemon fruits.

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INTRODUCTION

In agricultural production plant diseases caused by bacteria are a major economic liability. Bacterial diseases have been a major challenge for many disease controls. This tackle is direct results of pathogen changeability, high opportunity for mutation or gene transmit in the pathogen when confronted with resistance genes or bactericides, high pathogen duplication rate during optimal conditions for disease expansion, and lack of sufficient chemical-based approaches for control. Chemicals such as bactericides or plant activators where applicable, introgression of plant resistance genes, and biological control strategies.[1]

Till date thousands of bactericides have been developed for benefit of mankind. Bactericides are the chemical compound used to control bacteria on plant. Bactericides show better control on pest but it has some disadvantages such as accumulation of bactericides in soil which are slowly degradable and toxic. Due to continuous usage of bactericide most of the pathogenic bacteria have developed resistance to a number of currently available antimicrobial agents. Development of resistance in bacterial pathogen against bactericides have become a global problem. [2]. Plant pathogens are responsible for the major economic losses in agriculture. The failure of bacterial disease management in agriculture usually caused by several factors such as lack of effective bactericides against the diseases, high pathogen variability and high mutation rate also resulting in bacteria overcoming [3]. Bacteriophages are viruses that infect and replicate in prokaryotic cell rather than eukaryotic cells. Use of phages is much safer than use of Bacteriocides, so bacteriophage could be the promising approach towards fight against pathogenic bacteria. Bacteriophage are effective than chemotherapy because highly specific and very effective in lysing targeted pathogenic bacteria and safe.

Xanthomonas is a pathogen of Rutaceae family plant. It is a plant pathogen causes canker disease in lemon fruit, orange, sweet orange, grapes etc. Currently many bacteriocides are available to treat canker disease. Canker reduces the yield of fruit which causes economical loss. The conventional method for the management of this disease is use of this bacteriocide. But the emergence of resistance to bacteriocide and its deposition in soil are few limiting factors.

Specific phage infects the *Xanthomonas* and carry out its lysis which reduce further infection of pathogen and used as biocontrol agent. Different formulations protect phage from different environmental factor or harsh environment and increase its effectiveness. Reduced fruit spots incidence with biweekly spray application of phage suspension effective against *Xanthomonas*. The process involved isolating of phages active against the pathogen, screening them for host range and lytic ability, and selecting a lytic phage strain with the broadest host range for disease control.

Different materials have been studied to find out if they could lengthen the life of viruses following revelation to various physical factors. Skim milk formulation achieved the greatest reduction in disease 79% in the first experiment and 45% in the second one. Skim milk, pregelatinized corn flour and cascrete formulation, applied without phage, did not reduce the lension number [4] The phage could not eliminate *Xanthomonas* completely from seed, seed coat and cotyledon but phage treatment reduce number of bacteria. The degree of protection increased with increase in concentration of phage from 1:1 to 60:1 in ratio (phage:Xan) [5].

Efficiency of disease control in several instances has been affected by timing of bacteriophage applications relative to arrival of the pathogen. A marked reduction in bacterial spot seen only if phage treatment was applied one hour or one day before inoculation with the pathogen. There is clearly a need for identifying biologically inert formulations to improve efficacy of phages without enhancing ingress. Formulation increases longevity of phage. Application of bacteriophage in agriculture field can improve the quality, quantity and economy of crop production. This phenomenon used for control of chemical agent and it is ecofreindly.

In the view of above background, bacteriophages were isolated and their antimicrobial potential in the management of *Xanthomonas* infection of lemon was evaluated.

MATERIALS AND METHODS

a. Isolation of *Xanthomonas* species:

Xanthomonas sps. were isolated from infected lemon fruit using sterile nutrient agar plate and starr's agar medium (Yeast extract 10 g/l, Glucose

20 g/l, Calcium Carbonate 20 g/l, Agar 25 g/l, pH 6.5). The plates were incubated at 28ºC for 24 hrs. The cultures were stored at 4ºC.[5]

b. Phenotypic characterization of isolated species:

Identification was done with the help of Bergey's manual of determinative bacteriology. [6.7]

c. Infectivity assay:

Isolated pathogen was incubated on surface sterilized healthy lemon fruit by making aberration on fruit with surface sterilized cutter. After drying fruits were kept in sterile autoclaved bag and incubated for 72 hrs. *Xanthomonas* sps. was isolated from infected lemon fruit using sterile nutrient agar plate and Starr's agar medium. The plates were incubated at 28ºC for 24 hrs. The culture was stored at 4ºC.[8]

d. Collection of samples for isolation of bacteriophage:

Lemon farm soil is used as a source for isolation of phage soil sample collected from. 1 gm of soil was diluted in 10 ml distilled water and filtered through membrane filter assembly (filter size= 0.22µm). Collected filtrate was used to isolate bacteriophage. [9]

e. Isolation of bacteriophages:

Agar overlay techniques

Phages were isolated from phage suspension by the plaque assay technique. 0.1ml of mid log phase culture of *Xanthomonas* and 0.1 ml phage suspension was mixed in 7 ml sterile nutrient agar butt. Then overlaid on sterile nutrient agar plate (14.3 ml per plate). Plates were incubated at 28ºC for 48 hrs. [2,10]

f. Purification of bacteriophage:

Single plaque was transferred into 50 ml mid log phase culture of *Xanthomonas*. The flask was incubated at 28ºC for 4 days on incubator shaker. After incubation the broth was filtered by membrane filter assembly and seprate the lysate. [5,10 11]

g. Spot assay:

0.1ml of phage suspension was spotted on the lawn of (24hrs old) *Xanthomonas* and incubated at 28ºC for 24hrs. [510]

h. preparation of high titer phage stock

0.1ml of phage suspension was transferred into 50 ml mid log phase culture of *Xanthomonas.* The flask was incubated at 28°C for 2 days on incubator shaker. [5,10.12]

i. Determination of bacterial and plaque count

0.1ml of phage suspension and 0.1 ml of mid log culture of *Xanthomonas* was added in sterile nutrient broth (7ml) and plated on sterile plate and incubated at 28°C for 24 hrs. 0.1ml of 10^{-5} dilution of *Xanthomonas* was spread on sterile nutrient agar plate and incubated at 28°C for 24 hrs.[10,13,14]

j. Formulation of phage:

Bacteriophage specific for *Xanthomonas* was formulated with sucrose (5g/l) and skim milk (7.5g/l) separately.[8]

k. Application of formulated phage on detached leaves:

1µl of formulated phage suspension (*Xanthomonas* cells: phage = 2:1) were placed at each site on axial surface of detached leaves using inoculum infilteration techniques. Inoculated leaves kept in 0.5% agar plate (15-20 ml per100×15 mm petri dish) containing Ampicillin (32µg/ml).[15,16] Plates were kept in moisture chamber and the plate was incubated at 28°C for 2 days.

l. Detection of chlorophyll pigment:

After 2 days incubation chlorophyll pigment was detected by Arnon method to check the intensity of infection. [17,18,19] 1g of plant material crushed in mortal with pestle in minimum amount of 80% acetone. It was centrifuged at 5000rpm for 10 min. Supernatant was taken in 100 ml volumetric flask and final volume was adjusted with 80% acetone upto 100 ml. The flask was wrapped with black paper to prevent photo-oxidation. Absorbance was taken at 663nm and 645nm. [20,21,22]

Total chlorophyll was calculated using following formula

Total chlorophyll= $20.2\times A_{645}+8.02\times A_{663}$

Where A represent Absorbance

RESULT

a. Isolation of *Xanthomonas* species and Phenotypic characterization:

After 72hrs incubation at 28ºC colonies are observed on nutrient agar plate and Starr's agar plate

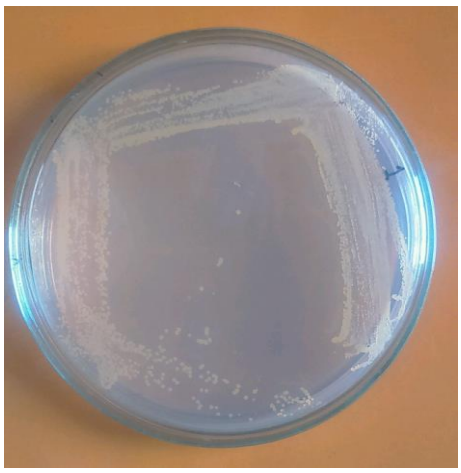


Fig 1: Isolated colonies on nutrient agar plate Isolated bacterial colony showed following characters

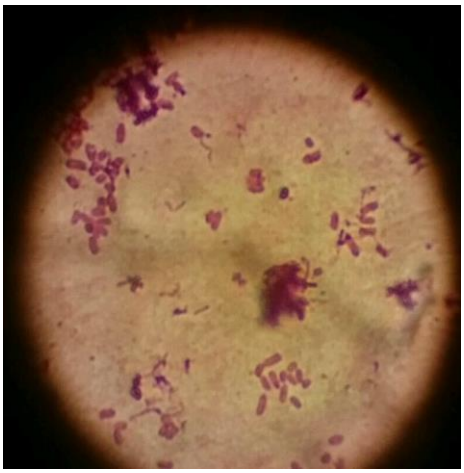


Fig 2 -Flagella staining

Using Bergey's manual isolate and biochemical tests was identified as *Xanthomonas*.

b. Infectivity assay:

After 72 hrs of incubation incubated region on lemon fruit show infection. Infected region shows small brown color canker.

Fig 3- Lemon re-infected with pathogen.

From infected lemon bacteria was isolated on nutrient agar plate and Starr's agar plate. Isolated bacteria show following characters. According to Bergey's manual isolated bacteria was identified as *Xanthomonas*.

c. Collection of samples for isolation of bacteriophage

Soil collected from the lemon farm was filter through sterile membrane filter assembly and filtrate was used for isolation of bacteriophage.

d. Isolation of bacteriophage:

After 48 hrs of incubation at 28ºC lysis of *Xanthomonas* cells indicates presence of bacteriophage,

	Size	Shape	Clear/Turbid
Soil sample	2mm	Circular	Clear

e. Multiplication of bacteriophage:

Single plaque inoculated in mid log phase culture of *Xanthomonas* showed reduction in optical density are as follows. Due to lysis turbidity of the suspension decreases. So, the OD of the suspension also decreases. Hence it was proved that there is presence of bacteriophages in the suspension.

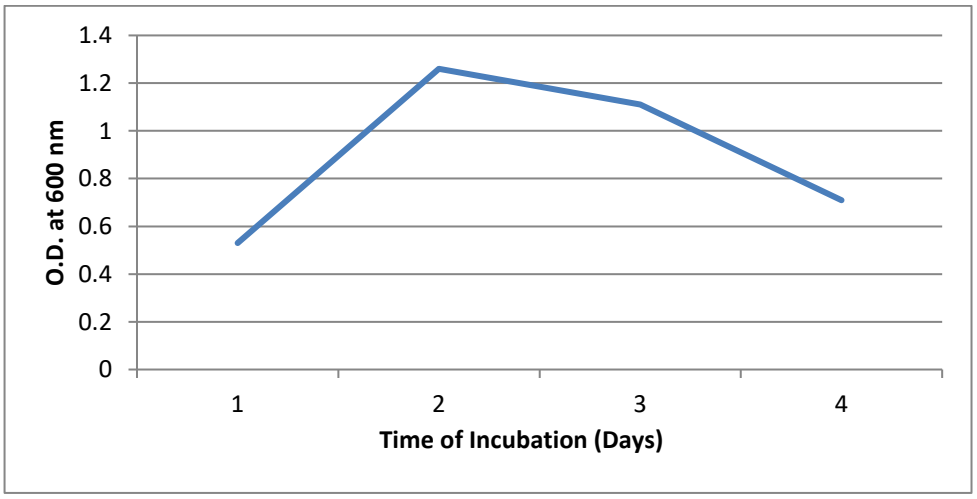


Fig 4: O.D.at 600nm of single plaque inoculated in mid log phase culture of *Xanthomonas.*

f. Spot assay:

spot of bacteriophage on lawn of *Xanthomonas* show clear and transperant spherical shape plaques after Incubatation for 24hrs.

g. Measurement of PFU and CFU

After 24 hrs of incubation 220 plaques per ml are observed

Plaque forming unit per ml was 220.

After 24 hrs of incubation 780 $\times 10^6$ colonies were observed.

Colony forming unit per ml was 780×10^6

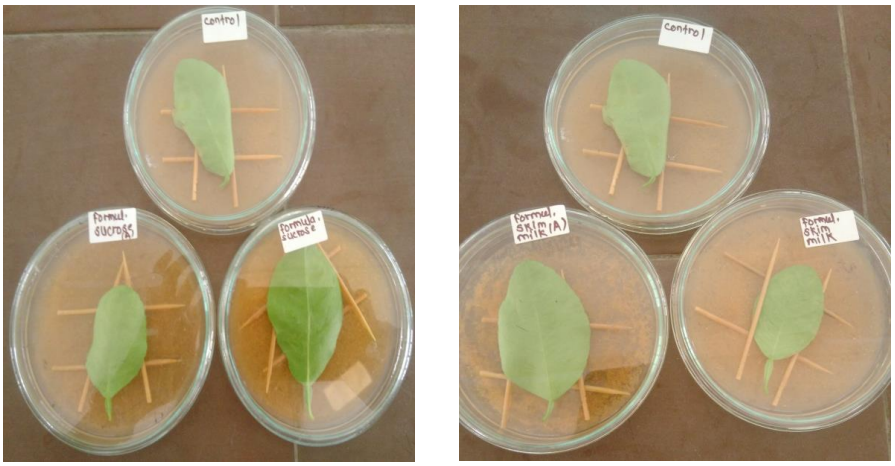
Fig 5- Grown culture plate of *Xanthomonas* showing Plaques.

Application of formulated phage lysate on detached leaves:

After 2 days of incubation at 28ºC following results are obtained.

Table no. 1- Lesion number per leaf on detached leaves sprayed with formulation.

Serial number	Formulation	Incubation time (infected lesion per leaf)	
		48hrs	72hrs
1	Control (Host)	6	8
2	Skim milk	2	4
3	Sucrose	3	3



(a) **(b)**

Fig 6- Lesion on detached leave
(a)Sucrose formulation with control
(b)Skim milk formulation with control

h. Detection of chlorophyll pigment:

By using Arnon method leaves are processed and O.D taken at 663 nm and 643 nm and following results are obtained

Table No. 2- Total chlorophyll pigment

Sr. No.	Formulation	O.D at		Total chlorophyll pigment (mg/g)
		645nm	663nm	
1	Leaves	0.290	0.727	11.68
2	Control	0.184	0.452	7.34
3	Skim milk	0.192	0.488	7.80
4	Sucrose	0.209	0.505	8.27

DISCUSSION

Xanthomonas species was isolated from the infected lemon fruit. It reinfect to lemon fruit and leaves which causes internal damage to plant which effect on yeild and quality of fruit. Which is responsible for the major economic loses in agriculture. [6,7,8]

Bacteriophage were introduced for control of bacterial canker on lemon plant. Bacteriophage showed lysis of *Xanthomonas* cell which reduces the spreading of disease. [10,11] Use of bacteriophage was effective than chemical control method because phage is not toxic for another living organism. Chemical agent accumulates in soil and polluted the soil. [12,13]

Different formulations of bacteriophage can be used to for control of xanthomonas infection. Phage lysate consisit of milk, sugar for alleviate the effect of light irradiation and provide rain-fastness. [1,15,16] Phages are incorporated with fertilizer because the agriculture production is carried out in open environment. By using phage alone, they can only deployed into the

system where there is little or no control over the environmental factors, such as temperature, light irradiation, moisture level and P^H.

Formulation that increase longevity of bacteriophage could improve the efficacy of phage treatment. Protective formulation of skim milk and sucrose was used. Sucrose formulation was effective to control canker disease caused by *Xanthomonas* sps[4].

In summary, our result indicated that the efficacy of phage treatment could be increased by the use of protective formulation. Neverthless, further research is needed in proper duration of application, formulation optimization, application frequency, phage dose and treatment. bactriophages could stand as a valid alternative to chemical bactericides for treatment of bacterial plant disease in the future.

CONCLUSION

Worldwide on food production have a serious impact of Plant diseases. Bacteriophages are ecofriendly compared to the other bacteriocide. Specific phage shows effective control disease which increase yeild and quality of fruit production.

Formulation of bacteriophage was found to increase longevity of bacteriophage and sucrose formulation was effective to control canker disease caused by *Xanthomonas* sps.

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REFERENCES

1. Tan G. and Tony P., Disease control of *Ralstonia solanacaerum* in tomato and *Xanthomonas campestris* in pitaya using bacteriophage. Department of Agriculture technology, (2014)
2. Cooksey D., Genetics if bacteriocide resistance in plant pathogenic bacteria. Ann. Rev phytopathol, 28: 201-219, (1990)
3. Agrios G., Plant pathology, 5th edition, Son Diego, Elsevier Academic press, PP. 4-9, (2004)
4. Balogh B., Improved efficacy of newly formulated bacteriophages for management of bacterial spot on tomato. university of Florida, (2002)
5. Boroh V., Jindal J. and Verma J., Biological management of bacterial leaf spot of mungbean caused by *Xanthomonas axonopodis* pv.*Vignaeradiatae* Division of plant pathology. Indian agricultural Research Institute, New Delhi 110 012, (2000)
6. Holt J., Krieg N., Sneath P., Staley J. and Williams S., Bergey's mannual of Determinative bacteriology, 9th Edn, Lippincott Williams & Wilkins:115-116, (2000)
7. Dye D., The inadequate of usual determinative tests for identification of *Xanthomonas* spp. N.Z.J sci 5:393 416, (1962)
8. Balogh B., Canteros B., Stall R. and Jones J.,Control of citrus canker and citrus bacterial spot with bacteriophage. plant Dis 92: 1048-1052, (2008)
9. McManus P., Stockwell V., Sundin G. and Jones A., Antibiotic use in plant agriculture. Annual Review of phytopathology, vo 40, PP 443-465, (2002)
10. Clokie M. and Kropinski A., Eds; Bacteriophages Methods and protocols, volume 1: Isolation, characterization and interaction. Humana press, New York, NY, USA, (2009)
11. Gill J., Hyman P., Phage choice, isolation and preparation for phage therapy. Curr pharm Biotechno.; 11: 2-10, PMID – 20214604, (2010)
12. McNeils D., Romero S., Kandula J., Stark C., Stewart A., & Larsen S., Bacteriophage: a potential biocontrol agent against wal nut blight (*Xanthomonas campestris* PV. Juglandis). New Zealand plant protection, vol 55 PP. 220-224, (2001)
13. Velidze A., Alavidae Z., and Glenn J. Bacteriophage therapy. Antimicrobe Agents the mother 45: 649-659, (2001)
14. Klement Z., Some new specific bacteriophage for plant pathogenic *Xanthomonas* spp. Natrure vol. 184, no.4694, pp. 1248-1959.
15. Randhawa P. and Civerolo P., A detached – Leaf Bioassay for *Xanthomonas compestris* Pv.pruni. friut laboratory, U.S. Deparment of Agriculture, Beltsville Agriculture Research center, Beltsville,MD 20705, (1985)
16. Obradovic A. and Jones J., Management of tomato bacterial spot in the field by foliar application of bacteriophages and SAR inducers, (2004)
17. Rajalkshmi. K., Extraction and estimation of chlorophyll from medicinal plant. Department of Biotechnology, Vels Institute of Science, Technology and Advanced studies, Chennai, Tamil Nadu, India, Volume 4, ISSN: 2319-7064, (2015)
18. Dubey R.and Maheshwari D., practical microbiology, 2nd Edn, S. Chand & Co. P Ltd, New Delhi, p. 413. ISBN: 81:219-2559-2, (2007)
19. Cappuccino J. and Sherman N "Microbiology-a Laboratory manual, 6th Edn, Pearson Education, (2004)
20. Okabe N. and Gota M., Bacteriophages of plant pathogen. Annual review of phytopathology, vol 1, PP-397-418, (1963)

21. Das M, Bhowniick T., Ahern S., Young R., Gonzalez C., Control of pierce's disease by phage. Department of plant Pathology and Microbiology, Texas A &M University,College Station, Texas, United State of America, (2015)

22. Lang J., Gent D.& Schwartz H., Management of *Xanthomonas* leaf light of onion with bacteriophages and a plant activator. Plant Disease, vol. gL, no.7, PP. 871-878, (2007)