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Biocontrol capability of bacteriophages against soft rot disease caused by *Dickeya chrysanthemi*

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ABSTRACT

Aims: This study aims to evaluate the effectiveness of bacteriophages isolated from Klang and Penang, Malaysia against *Dickeya chrysanthemi* that causes soft rot disease.
Methodology and results: Basic characterization such as dextrose test, citrate test, lactose fermentation test and ornithine test were carried out on *D. chrysanthemi*. Activity of bacteriophages against *D. chrysanthemi* was evaluated using spot test. Double agar overlay assay was performed to purify and enumerate the quantify of bacteriophages. Bacteriophages were also checked for its effectiveness in controlling soft rot on post-harvested vegetables: potato (*Solanum tuberosum*), cucumber (*Cucumis sativus*) and apple (*Malus domestica*). Results showed that *D. chrysanthemi* able to utilize citrate and dextrose as the source of energy, which indicated that *D. chrysanthemi* inclined to choose fruits and vegetables containing citrate and dextrose as the target of attack. Clear zone observed on the bacterial lawn (spot test) indicated the ability of the bacteriophages to infect and lyse *D. chrysanthemi.* All the bacteriophages studied herein reached the highest concentration on day 3 and were monovalent.
Conclusion, significance and impact of study: All the isolated bacteriophages were able to restrain the spreading of soft rot caused by *D. chrysanthemi* either work alone or as cocktail. This study provides information for the formulation development of bacteriophage against soft rot disease cause by *D. chrysanthemi*. Furthermore, this study reveals the potential of locally isolated bacteriophages against the *D. chrysanthemi* and paving the application of phage treatment on agriculture products that are not limited to potatoes, cucumber and apple.

Keywords: Bacteriophage, *Dickeya chrysanthemi*, soft rot disease, phage treatment

INTRODUCTION

Dickeya chrysanthemi formerly was named as *Erwinia chrysanthemi* (Palacio-Bielsa *et al.*, 2006; Sandy and Butler, 2011) belongs to the family Enterobacteriaceae (Samson *et al.*, 2005), also known as *Dickeya dadantii* (Grenier *et al.*, 2006; Condemine and Ghazi, 2007). *Dickeya chrysanthemi* is a Gram-negative bacterium vascular wilt pathogen. It colonizes the xylem and becoming systemic within a plant. It can remain latent in stock plants (ornamentals, bananas) and thus can be spread during the vegetative propagation process using cutting method (Dickey, 1981). It is reported that *Dickeya chrysanthemi* able to secrete pectin-degrading enzymes resulting in the disintegration of plant cell walls and subsequently degrading succulent fleshy plant organs such as roots, tubers, stem cuttings and thick leaves which causes problem of bacterial soft rot (Hugouvieux-Cotte-Pattat *et al.*, 1996).

Bacterial soft rot is a broad term that refers to many types of plant pathogens that cause plants to rot. It is one of the ten most critical problems in agriculture which limiting crop yields and quality (Czajkowski *et al.*, 2015). The infection of soft rot starts on the surface of injured stem or root and progresses inward. It is usually odourless in the early stage, but over time, the infected tissue is invaded by secondary organisms, which leads to the production of foul odour (Czajkowski *et al.*, 2011). Most symptoms are formation of watery lesions, tan colour and soft decay of the tissue surrounded by a dark brown to black ring. Some plants affected by these organisms exhibit wilting, stunting and chlorination (van der Wolf *et al.*, 1995). Numerous scientific researches have been carried out to combat this troublesome disease and the superior inhibitory performance of bacteriophage

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has been recorded (Lim *et al.*, 2013; Carstens *et al.*, 2018; Lee *et al.*, 2021).

In recent years, there has been a lot of researches using bacteriophage as a source of soft rot inhibition, but most researchers have focused on *Pectobacterium* spp. (Czajkowski, 2016; Zaczek-Moczydłowska *et al.*, 2020; Bugaeva *et al.*, 2021). Scholars have obtained satisfactory results on the treatment and control of soft rot caused by *Pectobacterium carotovorum* (Muturi *et al.*, 2019), *P. carotovorum* subsp*. carotovorum* (Lim *et al.*, 2013; Voronina *et al.*, 2019) and *P. atrosepticum* (Buttimer *et al.*, 2018; Carstens *et al.*, 2019). There are also studies on *Dickeya solani* (Adriaenssens *et al.*, 2012; Carstens *et al.*, 2018). However, the information exists about soft rot caused by *D. chrysanthemi* and lytic bacteriophage were only a handful. A study has reported the successful isolation of *Myoviridae* and *Siphoviridae* bacteriophages from Caspian Sea water that victoriously inhibit the growth of *D. dadantii* on cranesbill (*Geranium* spp.) (Soleimani-Delfan *et al.*, 2015). Similarly, *Sucellus* and *Amaethon* bacteriophages were also isolated form household organic waste sample at Denmark to target *D. dadantii* subsp. *dadantii* (NCPPB 4097) (Djurhuus *et al.*, 2020). Another study also reported phage ϕD5 isolated from arable field's soil at Pomorskie, Poland, expressed broad host range include *D. chrysanthemi* IPO2118 (Czajkowski *et al.*, 2014).

As *D. chrysanthemi* has indirectly contributed to damage of crops and agriculture economy, options solution available in combating this issue shall be explored widely to enable suppression of soft-rot diseases. Therefore, the purpose of this study was to evaluate the effect of bacteriophages isolated from Klang and Penang, Malaysia against soft rot caused by *D. chrysanthemi*.

MATERIALS AND METHODS

Characterization of *Dickeya chrysanthemi*

*Dickeya chrysanthem*i strain used in this study was obtained from American Type Culture Collection, United States. Lactose fermentation test (Trepeta and Edberg, 1984), citrate test (da Silva *et al.*, 2013), dextrose test and ornithine test (Thapa and Chapagain, 2020) were performed to explore the energy source of *D. chrysanthemi* (ATCC 11663). MacConkey agar (HIMEDIA, India) and potato dextrose agar (HIMEDIA, India) were served as medium for lactose fermentation test and dextrose test, respectively. Citrate test and ornithine test were conducted by subculture *D. chrysanthemi* (ATCC 11663) on Simmons citrate agar (HIMEDIA, India) and Motility Indole Ornithine (MIO) agar (HIMEDIA, India).

Lactose fermentation test, dextrose test, citrate test and ornithine test

MacConkey agar and dextrose agar were prepared by mixing 25.75 g of pre-mix MacConkey agar powder, 12 g of pre-mix potato dextrose broth powder and 7.5 g of agar powder in 500 mL of distilled water, respectively. Preparation of Simmons citrate agar and MIO agar were completed by dissolving 0.36 g of pre-mix Simmons citrate agar powder and 0.47 g of pre-mix MIO agar powder in 15 mL of distilled water, respectively. All mixtures were autoclaved at temperature of 121 °C for 15 min at 15 psi pressure and poured into Petri dishes. The prepared agars were inoculated with *D. chrysanthemi* (ATCC 11663) strain and incubated at 29 °C for 3 days. The final results (3 replicates) were observed with naked eyes.

Isolation of bacteriophages

Bacteriophages were collected from Butterworth sewage (Phage B) (5.43036, 100.39732), Klang sewage (Phage K) (3.12518, 101.44133) and market drain water (Phage M) (5.32912, 100.28634) by submerged a sterile glass bottle below the water surface. The collected samples were stored at 4 °C and further action was performed within 6 hours after collection. Bacteriophages that specific to *D. chrysanthemi* (ATCC 11663) were isolated and amplified using method described by Cerveny *et al.* (2002). A mixture of bacteriophage and strain was prepared by mixing 20 mL of collected sample, 20 mL of Luria Bertani (LB) broth with 2 mL of LB culture of *D. chrysanthemi* (ATCC 11663) strain, followed by incubated overnight in incubator shaker (New Brunswick Scientific, United States) at 29 °C. After overnight incubation, the mixture was centrifuged at 10000 rpm for 10 min and filtered through 0.22 µm syringe filter. After that, 20 mL of the prepared mixture were mixed with an equal volume of *D. chrysanthemi* (ATCC 11663) culture. The resultant mixture was once again incubated, centrifugated and filtrated as described above. The final filtrate was stored at 4 °C for subsequent use in the study.

Spot assay

Spot assay was performed to detect phage activity. To do this, a broth culture of *D. chrysanthemi* (ATCC 11663) was prepared. This is done by using single colony of *D. chrysanthemi* (ATCC 11663) that growing on Luria Bertani (LB) agar (Friendemann Schmidt, Australia) and transferred into a sterile universal bottle containing 10 mL fresh LB broth and incubated overnight at 29 °C in incubator (MEMMERT, Germany). Then, lawn culture was prepared (Burdass *et al.*, 2016). The plate was kept at room temperature until the spreading culture dried out and use a marker to divide four equal spaces on the back of the plate. Separately, 20 µL of distilled water and three bacteriophage samples were pipetted on the divided bacterial lawn, the plate was incubated overnight at 29 °C. Clear zones were checked after 18 h of incubation (Rahaman *et al.*, 2014).

Double agar overlay assay

Double agar overlay assay was performed to obtain

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homogenous plaques. In brief, 100 µL of bacteriophage was mixed with 100 µL of bacterial culture in a test tube containing 3 mL of soft agar (0.8% agar). The mixture was then poured onto an agar plate containing LB agar. The soft overlay agar was allowed to solidify at room temperature and placed inverted follow by overnight incubation at 29 °C (Rahaman *et al.*, 2014; Rahimzadeh *et al.*, 2016b).

Purification of bacteriophages

Lytic plaques with the same morphology were collected from the overlay agar using sterile micropipette tip and suspended into 10 mL culture of *D. chrysanthemi* (ATCC 11663). The mixture was then incubated overnight at 29 °C in an incubator shaker. The suspension was centrifuged at 10000 rpm for 10 min at 4 °C and the supernatant was filtered with 0.22 µm syringe filter which was then proceeded with double agar overlay assay as described above. This process was repeated thrice (Rahaman *et al.*, 2014).

Determination of phage titer

Phage titer (concentration of bacteriophage) was determined to enumerate the time when highest quantity of bacteriophages was achieved through plaques formed on the agar plate. The numbers of plaques formed on the agar were counted as plaque forming unit (PFU) and calculated using the equation below.

Phage titer = Number of plaques/(Volume of phage added × Dilution factor)

Details of the procedures were presented as *Part I* and *Part II* below:

Part I

Ten-fold serial dilution of bacteriophage was prepared by mixing 100 µL of each dilution with 900 µL aliquots of LB broth, from 10^{-1} to 10^{-10} (Rahimzadeh *et al.*, 2016a). Pre-inoculum bacterial culture (300 µL) was then added to the diluted bacteriophage of each fold, mixed well and incubated at 29 °C for 15 min. Succinctly, 1 mL of the mixture was mixed with 3 mL of soft agar (0.8% agar) to carry out double agar overlay assay.

Part II

After incubation, one to two plaques were transferred from overlay agar into 4 separate universal bottles containing 10 mL culture of *D. chrysanthemi* (ATCC 11663). The mixtures were incubated at 29 °C in an incubator shaker. At an interval of 24 h, 48 h, 72 h and 96 h, one bottle was removed and stored at 4 °C, respectively. All the samples were centrifuged at 10000 rpm for 10 min at 4 °C and the supernatant was filtered with 0.22 µm syringe filter. All the four filtrates were subjected to repeat the steps mentioned in *Part I*.

Sample with more plaques represented higher phage titer. Plates with more than 300 plaques were recorded as too many to count (TMTC), while plates with less than 30 plaques were recorded as too few to count (TFTC).

Host range determination

Bacteriophages host range test was performed on the following: *Dickeya chrysanthemi* (ATCC 11663), *Erwinia carotovora* (ATCC 39048), *Xanthomonas vesicatoria* (ATCC 35937) and *Xanthomonas gardneri* (ATCC 19865) with 3 replicates. Lawn culture of each bacterial were prepared using the method described previously (Burdass *et al.*, 2016). Twenty microliters of bacteriophage was pipetted onto the lawn culture and let dry before overnight incubation at 29 °C. The plates were checked after 18 h for the presence of clear zone of lysis.

Effectiveness of bacteriophages in controlling soft rot on post-harvested vegetables

Healthy post-harvest vegetables (*Solanum tuberosum*, *Malus domestica* and *Cucumis sativus*) were selected as assay samples. The vegetables were divided into seven experimental groups with 3 replicates (Table 1).

All the vegetables were washed under running tap water to remove soil particles, sterilized with 70% ethanol and rinsed with distilled water to remove excess ethanol. The vegetables were air-dried for 30 min on a sterile surface and cut into suitable size that were placed in sterile Petri dishes. One hole was made using pipette tip on the vegetable's surface served as the site of infection. After that, 50 µL of bacterial suspension was inoculated into the hole on the surface of vegetables and left to stand for 10 min to allow the fluid to diffuse into the tissues. Next, 200 µL of bacteriophage solution: Phage B (8.6×10^{10} PFU/mL), Phage K (6.4×10^{10} PFU/mL), Phage M (9.5×10^{9} PFU/mL) was applied on the infected site and the experimental vegetables were covered with plastic bag and kept at room temperature for 5 days. The area of lesion was measured and recorded.

Table 1: The experimental groups on post-harvested vegetables.

Group	Sample used
a	Inoculated with distilled water
b	Inoculated with phage cocktail
c	Inoculated with *Dickeya chrysanthemi* (10^{8} PFU/mL) only
d	Inoculated with bacteria + Phage B (8.6×10^{10} PFU/mL)
e	Inoculated with bacteria + Phage K (6.4×10^{10} PFU/mL)
f	Inoculated with bacteria + Phage M (9.5×10^{9} PFU/mL)
g	Inoculated with bacteria + Phage cocktail

Phage cocktail = Phage B (8.6×10^{10} PFU/mL) + Phage K (6.4×10^{10} PFU/mL) + Phage M (9.5×10^{9} PFU/mL)

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Statistical analysis

All values were reported as the mean ± SEM (standard error of mean) and were analyzed by one-way ANOVA using Social Sciences software (SPSS Version 27), $p<0.05$ was considered as significance.

RESULTS

Characterization of *Dickeya chrysanthemi*

Fermentation in microorganisms is referring to degrading organic nutrients for production of adenosine triphosphate (ATP) (Prescott *et al.*, 2004). Colonies on MacConkey agar appear to be colourless and the surrounding agar of the colonies remains relatively transparent indicated that *D. chrysanthemi* (ATCC 11663) was non-lactose fermenter (Figure 1a). Light brownish colonies grew on the light amber colour clear agar surface after 3 days of incubation proved that the bacteria were able to ferment dextrose for energy production (Figure 1b). As the bacteria utilized citrate as carbon source, the medium of citrate test change from green to royal blue (Figure 2a). Ornithine test showed a negative result as resulting of yellow colour in the medium (Figure 2b).

Spot test

As shown in Figure 3, clear zone was formed on *D. chrysanthemi* (ATCC 11663) bacterial lawn. This indicated that all 3 bacteriophages exhibited antibacterial activity against *D. chrysanthemi* (ATCC 11663).

Phage titer determination

Figure 4 shows the phage titer for all 4 days of the titer determination for all the 3 bacteriophages isolated. On day 1 and day 2 the titer of Phage B and K didn't show significant different, while Phage M was significantly lower than Phage B and K ($p<0.05$). On day 3, there was significant different of phage titer among all the three isolated bacteriophages ($p<0.05$). Specifically, the highest titer was observed on day 3 for all the bacteriophages which is, 8.6×10^{10} PFU/mL for Phage B, 6.4×10^{10} PFU/mL for Phage K and 9.5×10^{9} PFU/mL for Phage M.

Host range determination

Result from the spot assay shows narrow host range, all 3 bacteriophages were only able to infect and lyse *D. chrysanthemi* (ATCC 11663) which indicate their specificity (Table 2).

Effectiveness of bacteriophages in controlling soft rot on post-harvested vegetables

The experimental result excellently proved the effectiveness of isolated bacteriophages in control soft rot caused by *D. chrysanthemi* (ATCC 11663). There was no lesion area on negative control experiment group a (Figure 5Ia, 5IIa and 5IIIa) and group b which only inoculated with phage cocktail (Figure 5Ib, 5IIb and 5IIIb). While in experimental group c (Figure 5Ic, 5IIc and 5IIIc) with only bacteria inoculation showed largest soft rot area.

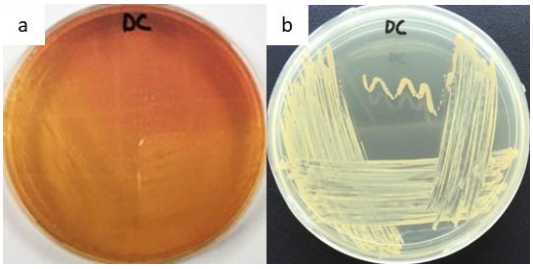

Figure 1: Growth of colourless colonies on MacConkey agar (a) shows that *Dickeya chrysanthemi* unable to ferment lactose. Growth of light brownish colonies on potato dextrose agar (b) indicated that *D. chrysanthemi* was able to use dextrose as one of the sources of energy.

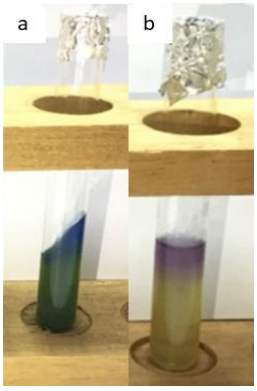

Figure 2: Positive citrate test result shows through the royal blue formed on the top of Simmons Citrate agar (a), which indicate the ability of *Dickeya chrysanthemi* to ferment citrate; yellow colour appeared in between of MIO agar (b) represented the failure of *D. chrysanthemi* to utilized ornithine as energy source.

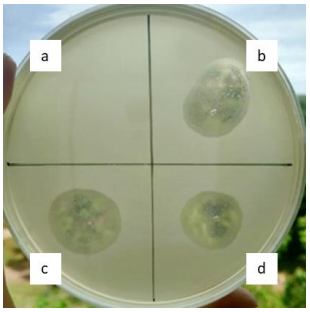

Figure 3: Spot assay which shows activity of bacteriophages against *D. chrysanthemi.* (a) Sterile distilled water (b) Phage K – 6.4×10^{10} PFU/mL (c) Phage B – 8.6×10^{10} PFU/mL (d) Phage M – 9.5×10^{9} PFU/mL.

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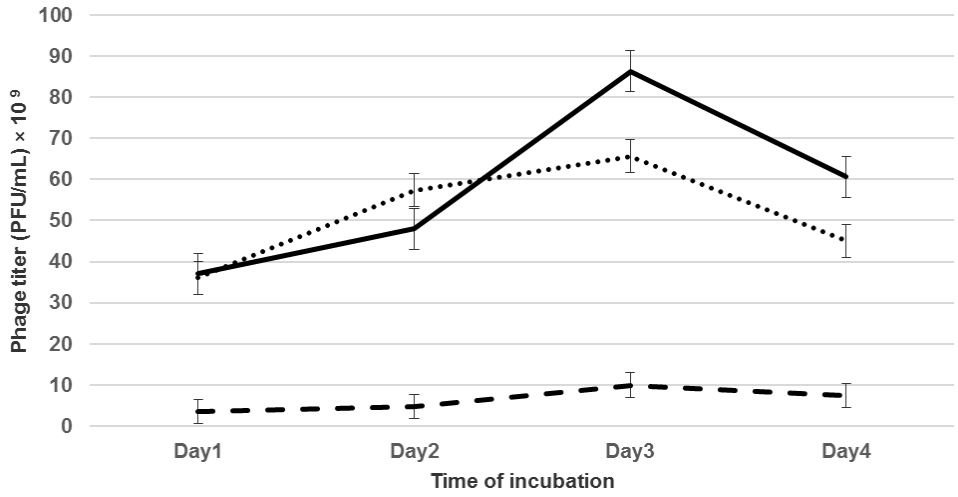

Figure 4: Titer of all the 3 isolated bacteriophages. As shown in the figure, all the isolated bacteriophages reached their highest titer on day 3 (——— Phage B, ····· Phage K, ------ Phage M).

Table 2: Host range determination result.

Bacteria	Phage B	Phage K	Phage M
Dickeya chrysanthemi (ATCC 11663)	10 ± 2 mm	12 ± 1 mm	8 ± 2 mm
Erwinia carotovora (ATCC 39048)	-	-	-
Xanthomonas vesicatoria (ATCC 35937)	-	-	-
Xanthomonas gardneri (ATCC 19865)	-	-	-

-: no clear zone.

Table 3: The inhibition results of various single bacteriophages and cocktail against soft rot disease on post-harvested vegetables.

Group	Soft rot area/Lesion area (cm^2)		
	Malus domestica	*Cucumis sativus*	*Solanum tuberosum*
a	x	x	x
b	x	x	x
c	5.617 ± 1.045	18.613 ± 1.161	4.811 ± 0.965
d	1.195 ± 0.113*	1.272 ± 0.298*	1.772 ± 0.235*
e	1.328 ± 0.209*	1.732 ± 0.702*	1.858 ± 0.373*
f	1.272 ± 0.298*	1.743 ± 0.698*	2.026 ± 0.448*
g	1.473 ± 0.254*	1.879 ± 0.616*	2.662 ± 0.585*

Values are expressed as mean ± SEM. Data was analyzed statistically using one way ANOVA. *Statistically significant $p<0.05$ as compared to group c (untreated). x, no lesion; a, inoculate with distilled water (negative control); b, inoculate with phage cocktail; c, inoculate with bacteria only; d, inoculate with bacteria and phage B; e, inoculate with bacteria and phage K; f, inoculate with bacteria and phage M; g, inoculate with bacteria and phage cocktail.

Application of bacteriophages (work alone or as a mixture of cocktail) on post harvested vegetables with bacterial pathogen inoculation resulted in a reduction of lesion area (Figure 5Id–5Ig, 5IId–5IIg and 5IIId–5IIIg). The experimental data manifested that the isolated bacteriophages were able to suppress soft rot on the infected vegetables and fruit ($p<0.05$) (Table 3). Based on the statistical data, all the three bacteriophages shown significant result ($p<0.05$) as single treatment or work together as cocktail compared to the untreated (group c).

DISCUSSION

Soft rot disease causing agricultural losses is growing concern in Malaysia. Besides the frequently cited pathogenic bacteria *Pectobacterium wasabiae* (Golkhandan *et al*., 2013) and *P. carotovorum* subsp. *carotovorum* (Nazerian *et al.*, 2011), this study looks into the soft rot disease caused by *D. chrysanthemi* (ATCC 11663). In an effort to provide biological disease control measures, 3 bacteriophages were

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Figure 5: Soft rot inhibition results on post-harvested vegetables. (a) inoculate with distilled water; (b) inoculate with phage cocktail; (c) inoculate with bacteria only; (d) inoculate with bacteria and phage B; (e) inoculate with bacteria and phage K; (f) inoculate with bacteria and phage M; (g) inoculate with bacteria and phage cocktail.

successfully isolated locally in Malaysia which showed satisfactory result in lysing the bacteria studied (Figure 3). It is worth mentioning that titer of the obtained bacteriophages was the highest on the day 3 of incubation. This finding may provide useful information on the development of bacteriophage spray application during post-harvesting (Balogh *et al.*, 2003; Choińska-Pulit *et al.*, 2015; Rosner and Clark, 2021).

Host range is an important reference index for understanding the pathogenicity and epidemiology of pathogen (McLeish *et al.*, 2018). Viruses with narrow host range (monovalent) are usually restricted to only infect and replicate within members of a single species (Kalmanson *et al.*, 1942; Suttle, 2000). In contrast, viruses with broad host range (polyvalent) are unlikely to rely on specific hosts for replication. In this study, three Gram-negative plant pathogen bacteria strains that have been reported to cause soft rot disease were included. These include *Erwinia carotovora* (Basset *et al.*, 2000), *Xanthomonas vesicatoria* and *X. gardneri* which was reported to cause bacterial leaf spot (Thieme *et al.*, 2005; Rashid *et al.*, 2015). The isolated bacteriophages only showed its lytic activity specific to *D. chrysanthemi* (ATCC 11663) (Table 2). The narrow host range of the bacteriophages identified herein can thus prevent from killing other species and maintaining the integrity of the host's microbiome (Kutter, 2009; Hyman, 2019).

Earlier biochemical tests showed that dextrose (Figure 1b) and citrate (Figure 2a) were the energy sources of *D. chrysanthemi* (ATCC 11663) which is further supported by other published studies (Huang *et al.*, 2010; Végh *et al.*, 2014). Therefore, potato, cucumber and apple were selected as experimental sample for soft rot infection in this study (Lu *et al.*, 2001; Yebra-Biurrun, 2005; Kirimura *et al.*, 2011; Ma *et al.*, 2018). To recap, bacteriophages in this study have shown effectiveness in restraining the soft rot progression. Similar investigation has also been reported for potato, which was using a different phage, i.e., LIMEstone1 and LIMEstone2 bacteriophages against *D. solani* (Adriaenssens *et al*., 2012). Many studies have cited that phage cocktail was more effective compared to single phage suspension (Chadha *et al.*, 2016; Costa *et al.*, 2019; Naghizadeh *et al.*, 2019; Yang *et al.*, 2020). These include the wider host range coverage and increase killing efficiency (Chan *et al.*, 2013; Fischer *et al.*, 2013; Rahimzadeh *et al.*, 2021). For instance, the pathogenicity of *Pectobacterium* in soft rotted potatoes was reduced using a cocktail conjugated of 5 bacteriophages (Bugaeva *et al.*, 2021). However, in the current study, the combination of Phage B, K and M, i.e., cocktail that investigated herein did not significantly inhibit the soft rot in comparison to the corresponding individual bacteriophages. This indicates there is no synergistic effect seen in the phage cocktail of the current study. One plausible reason for this observation may be due to the lytic mechanisms of the individual bacteriophages onto *D. chrysanthemi* (Chen *et al.*, 2018).

Treatment of the isolated bacteriophages on post-harvested vegetables provided effective control and limiting the spreading of soft-rot disease caused by *D. chrysanthemi* (ATCC 11663). This suggested that the bacteriophages can be applied for protecting vegetable and fruit against soft rot caused by *D. chrysanthemi* (ATCC 11663) as soon as after harvest or before storage. In addition, bacteriophage can be inoculated during the growth of fruits and vegetables to protect them from soft rot infection. However, since the stability of bacteriophage is affected by environmental factors, including temperature (Fister *et al.*, 2016), appropriate formulations will be required to keep the bacteriophage's viability prolonged after inoculation.

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CONCLUSION

This study shown the capability of *D. chrysanthemi* in fermenting dextrose and citrate as sources of energy. Therefore, plants containing substances that can serve as an energy source for *D. chrysanthemi* are more likely to be its targeted in order to conveniently generate energy during replication. Bacteriophages harvested from sewage and market drainage were able to specifically inhibit the soft rot caused by *D. chrysanthemi*. This is further evidenced by the phage treatment on post-harvested vegetables whereby all the three bacteriophages were shown to significantly limit the spreading of soft rot disease alone or in a mixture (cocktail).

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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