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Full Length Article

Partial Characterization of Bacteriophages from Indonesia and its Potency as Biocontrol of *Xanthomonas oryzae* pv. *oryzae*

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Abstract

Bacterial leaf blight (BLB) is a disease caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo) of rice in rice-producing countries including Indonesia and attack rice in all stages of growth. In the advanced, crop production will be decreased by up to 50–70%. Recently, the practical efforts to overcome the problem by using resistant varieties, antibiotics, and sanitation; however, the ability of the pathogen to forms the new virulent pathotypes is noteworthy. Alternatively, the pathogen could be environmental-friendly controlled by utilizing bacteriophages as biological control agents because of their specific characteristics to their bacterial hosts. This research aimed to obtain information about the characteristic of the first isolated bacteriophages from Indonesia. The result showed that two bacteriophages had been isolated from soil in Arjasa Jember and soil in Gadingan Situbondo, namely phage XooX1IDN and phage XooX2IDN, respectively. The two phages were inactivated at 80ºC and stable at pH within the range of 6 to 8. The phage XooX1IDN has a genome size of approximately 39 kb, while phage XooX2IDN had a genome size 38 kb. Morphologically, both phages possessed the family of *Myoviridae.* Phage biocontrol in vitro assay showed that both phages significantly reduced the growth of BLB pathogen, indicating that both phages potentially, as biological control agents for BLB disease in rice. © 2021 Friends Science Publishers

Keywords: Bacterial leaf blight; Phage therapy; *Myoviridae*; *Xanthomonas oryzae*

Introduction

Xanthomonas oryzae pathovar (pv.) *oryzae* (Xoo) is a Gram-negative bacterium found in field of rice-producing countries including Indonesia. This bacterium is a causative agent of bacterial leaf blight (BLB), a destructive bacterial disease that is prevalent among various rice varieties in the rice growing countries including Indonesia (Singh *et al*. 2015). Since the pathogen multiplies in xylem and predominantly invades the vascular tissue, the most common symptom of this disease is wilting, especially in young leaves namely "Kresek" disease and decrease rice production (Nino-Liu *et al*. 2006). BLB remains a serious problem on rice production, especially in Asia where the infection of pathogen results in enormous losses of yield ranging 6 to 90 percents in some rice varieties (Singh *et al*. 1980; Bhutto *et al*. 2018).

Numerous studies have reported the management strategies of bacterial leaf blight such as chemical control, genetic resistance, and biological control (Kim *et al*. 2016). A number of studies have reported plant genes that confer resistance against *X. oryzae* through the plant breeding using series of resistance gene (R genes), designated from the Xa genes of rice cultivars (Degrasi *et al*. 2010). Unfortunately, this strategy is ineffective due to the ability of BLB pathogen to form a new and more virulent pathotypes because of Xoo's diversity and gene mutation mechanism of *X. oryzae* to breakdown the resistance genes of rice (Keller *et al*. 2000; Ponciano *et al*. 2003; Shanti *et al*. 2010). Biological control thus seems to be an alternative way to manage this disease being cost-effectively, sustainable and eco-friendly (Gnanamanickam 2009). Among the alternative of biological control agent, the use of bacteriophage could be a promising control technique, known as phage therapy (Addy *et al*. 2012a).

Bacteriophage is a virus that infects and multiplies within bacterial host cells, causing lysis along with the development of bacteriophage particles in specific host cells, and attacks a narrow bacterial strain (Beaudoin *et al*. 2007). Recently, the use of the phage as an approch to control bacterial pathogens has been highly attractive since some reports proved the potency of phage to control it

bacterial host (Svircev *et al*. 2018). Ralstonia phage RsoM1USA has been found to have ability to inhibit the growth of Ralstonia solanacearum, a bacterial wilt pathogen on several crops (Addy *et al*. 2019). Moreover, Ahmad *et al*. (2014) isolated CP1 and CP2 bacteriophages that were able to control *X. axonopodis* pv *citri* on citrus. Mostly, bacteriophage can be easily isolated from the soil and irrigation water around infected crops (Bielke *et al*. 2012; Kalpage and Costa 2014) and from the symptomatic plant parts (Ritchie and Klos 1977).

Although, bacteriophage is easy to explore; however, the selection of bacteriophage isolates become crucial point in exploitation of bacteriophage for phage therapy (Addy *et al*. 2012a; Svircev *et al*. 2018). It is because bacterial host cells exhibit the changes in virulence after infection by the phage such as production of plant toxin and increase in virulence factors (Verheust *et al*. 2010). For example, infection of Ralstonia phage RSS1 increases the virulence of *R. solanacearum* to be more destructive on tomato (Addy *et al*. 2012b). In contrast, phage XacF1 decreases the virulence of *Xanthomonas axonopodis* pv *citri* to infect citrus leaves (Ahmad *et al*. 2014).

Several studies have been reported to explore bacteriophage as biological control agent of *X. oryzae* pv. *oryze*. About 10 bacteriophages have been isolated from Vietnam and Thailand (Kovács *et al*. 2019), China (Dong *et al*. 2018), Japan (Kuo *et al*. 1967) and India (Ranjani *et al*. 2018). None of the study has been reported on the bacteriophage of *X. oryzae* isolated from Indonesia. Therefore, this study is aimed to explore the bacteriophage as an initial step prior to its use as biological control agents for the first time from Indonesia.

Materials and Methods

Bacterial strain

Xanthomonas oryzae XooJ2 was isolated from the infected rice leaves (56-day-old plant after transplanting) in the rice field showing "Kresek" symptoms and was routinely cultured on yeast extract dextrose agar (YDA) at 28°C for 72 h. The bacterium was confirmed through several biochemical tests such as the KOH solubility assay, catalase test, starch hydrolysis assay, and pathogenicity test using cultivar Logawa (Schaad *et al*. 2001). In addition, confirmation was done by detecting the presence of specific gene sequence in *X. oryzae* pv. *oryzae* was done through polymerase chain reaction (PCR) using specific pair primer of JLXoo-230F (5'-CCT CTA TGA GTC GGG AGC-3') and JLXoo-230R (5'-ACA CCG TGA TGC AAT GAA GA -3'). The GoTag PCR mixture (Promega, USA) was subjected to a 35 cycles after pre-denaturation at 96°C for 5 min, followed by denaturation at 96°C for 1 min, 55°C for 3 min, 72°C for 1 min, and a final extension step at 72°C for 7 min. The PCR product was subjected to gel electrophoresis in a 1.5% (wt/vol) agarose gel in TAE, followed by staining with ethidium bromide (Lu *et al*. 2014).

Isolation and purification of xanthomonas infecting bacteriophages

One gram of soil samples, collected from rice fields in District Arjasa, Regency, Jember and District Gadingan, Regency Situbondo, East Java Province, Indonesia, were used for phage isolation using the basic enrichment method (Addy *et al*. 2019). Briefly, soil sample was suspended with 2 mL of sterile water and shaken for 24 h. One milliliter of suspension was taken and filtered through 0.45-µm membrane filter (Steradisc, Krabo Co., Japan) and use as phage lysate in plaque assay with XooJ2 as host. Bacteriophages were then purified as described by (Ahmad *et al*. 2017). Routinely, 24 h-old bacterial culture was used as host for phage's propagation. Pure bacteriophage particles were stored at 4°C until used in further testing (Addy *et al*. 2019). The morphology of phages was assessed by transmission electron microscopy.

Nucleic acid digestion and protein profile

To determine the nucleic acid type of bacteriophages, the genome of bacteriophages was digested with *Eco*RV restriction enzyme according to the supplier's instructions (Promega, USA). Eight microliters of phage DNA suspension was mixed with 9.5 μL sterile distilled water, 2 μL enzyme buffer and 0.5 μL restriction enzyme (*Eco*RV or *Xba*I) the mixture was incubated at 37°C for 60 min. DNA fragments were subjected to gel electrophoresis in 1% agarose gel.

To determine the protein profile, whole phage particles were subjected to Sodium Dedoxyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis. Briefly, whole phage particles were harvested using ultracentrifuge (Hitachi, Japan) at 4°C, 30.000 × g for 2 h and equal volumes of sample buffer (0.5 *M* Tris-HCl (pH 7.2) buffer containing 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.1% bromophenol blue) was added. The samples were boiled for 5 min. Gel was then stained and visualized using Coomassie brilliant blue dye.

Host specificity assay

To determine the host specificity of phage XooX1IDN and XooX2IDN, the purified phage was subjected to spot testing using XooJ2 and *R. solanacearum* DT3 as the bacterial target. In this test, three microliters of the phage suspension (10^3 PFU/mL) was spotted on top of the double-layered YDA plate. The formation of a clear zone on the spotting area indicated that the bacterium were susceptible to the phage. Potentially susceptible strains were tested further by serial dilution plaque assay to determine whether they were truly susceptible to the phage (Ahmad *et al*. 2017).

Bacteriophages stability assays

Xanthomonas phages were tested for their stability against

environmental factors such as temperature and pH (Iriarte *et al*. 2007). To determine the effect of temperature on the stability and infectivity of bacteriophages, the purified phage particles in SM buffer were incubated at different temperatures, 30°C to 80°C. While to determine the effect of pH, bacteriophage particles in SM buffer was adjusted to reach various pH of 3 to 9 followed by incubation at room temperature. Phage number was estimated by calculating plaque on the YDA plate using isolate XooJ2 as a host.

Biological control assay *in vitro*

To determine the effect of phages on XooJ2 (susceptible host), the growth of XooJ2 in NB medium (in 24-well plates) at 28°C was monitored on the phage XooX1IDN- and XooX2IDN-treated and untreated XooJ2. Briefly, the concentration of the overnight culture of XooJ2 was adjusted with NB to initial OD_{600} of 0.3, and 1.5 mL of the bacterial suspension was added to each well of the 24-well plate. One hundred and fifty microliters of phage suspension was then added at m.o.i of 0.01, 0.1, 1.0, and 10, respectively, and the plate was incubated inside Microplate reader SH-1000 (Corona Electric, Japan) with slow shaking. SM buffer was used as a phage control (m.o.i of 0). Bacterial growth was estimated by measuring the absorbance at 600 nm every 180 mins for 36 h. This experiment was repeated three times with three replications for each m.o.i treatment (Addy *et al*. 2019).

Results

The bacterial leaf blight pathogen

The isolate XooJ2 was isolated from 56-day-old rice from the symptomatic leaf of bacterial leaf blight in Jember. The isolate XooJ2 was purified and characterized by its biochemistry and molecular properties. The bacterium XooJ2 exhibited yellow, round in shape, convex, smooth surface, and flat edge colonies when grown on Nutrient Agar (NA) media (Fig. 1A). Furthermore, the genome of XooJ2 was subjected to PCR amplification using the *Xanthomonas oryzae* pv. *oryzae* specific PCR primer and resulted in the predicted band with an approximate size of 230 bp (Fig. 1B). The isolate XooJ2 also produced leaf blight symptoms after re-inoculation to the rice leaf (Fig. 1C).

Morphology plaques and phages, nucleic acid, and protein profile

Phage XooX1IDN and XooX2IDN, isolated from rice fields in Jember and Situbondo, showed turbid plaques (diameter 2 ± 1 mm) on tested medium (Fig. 2). Transmission electron microscope revealed similar tailed forms of both phages (Fig. 3). Analysis of protein bands patterns through SDS-PAGE showed that all bacteriophages had a similar composition of more than 10 sub-units of protein (Fig. 4A).

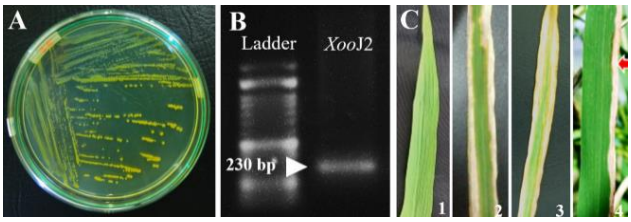

Fig. 1: Partial characteristic of bacterial host XooJ2, a pathogen of bacterial leaf blight on rice. XooJ2 colonies on YDA medium exhibit yellow colonies (**A**), agarose gel electrophoresis of PCR product of 230 bp using specific pair primer (**B**), and The leaves exhibit bacterial leaf blight symptoms in the field and the result of the reinoculation assay (**C**)

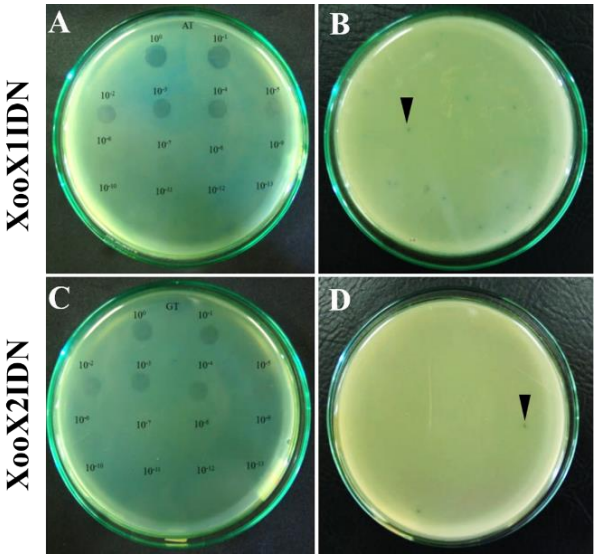

Fig. 2: Plaques morphology of phage XooX1IDN and XooX2IDN on tested media

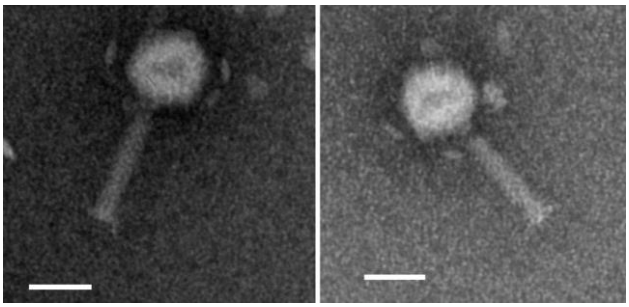

Fig. 3: Transmission electron microscopy of negatively stained (**A**) XooX1IDN and (**B**) XooX2IDN particles at 50-k fold magnification and at an acceleration voltage of 80 kV. Scale bar represents 50 nm

The genome of both bacteriophages of non-digested endonuclease was more than 10,000 bp and was clearly digested with DNAse and endonuclease restriction enzymes, but not RNAse (Fig. 4B). Moreover, *Eco*RV restriction enzyme provided similar patterns except for the particular

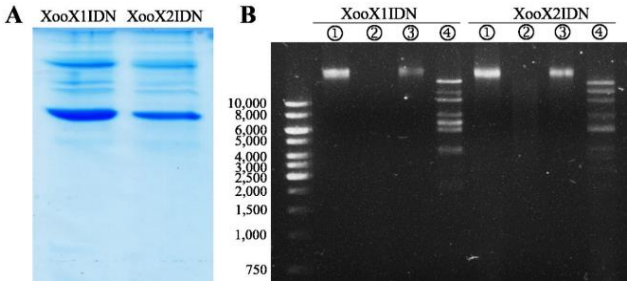

Fig. 4: Analysis of phage XooX1IDN and XooX2IDN characteristics. (**A**) Structural protein profile of phage particles on SDS-PAGE, (**B**) Restriction profile of phages nucleic acid (1) after digestion with DNAseI (2), RNAseA (3), and endonuclease *Eco*RV (4)

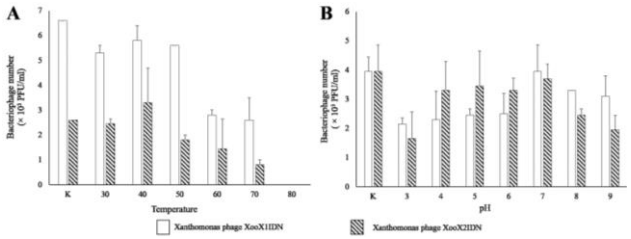

Fig. 5: Effect of (**A**) temperature and (**B**) pH on the phage XooX1IDN and XooX2IDN particles stability

band around 7.0 kbp (Fig. 4B, lane 4) and was predicted to have a genome size of approximately about 39 kbp for phage XooX2IDN and about 38 kb for phage XooX2IDN.

Effect of temperature, pH and host specificity

Some environmental factors such as temperature and pH contribute to the inactivation of bacteriophage particles. The result showed that the number of phage XooX1IDN and XooX2IDN particles began to decrease after incubation of both phages at 60°C and no bacteriophage particles were detected by incubating the particles at 80°C (Fig. 5A). Moreover, the phage XooX1IDN and XooX2IDN still formed plaques although the particles have pre-incubated in suspensions of different pH levels (Fig. 5B). In addition, both phages, XooX1IDN and XooX2IDN only formed plaques on XooJ2 lawn but not on *R. solanacearum* DT3.

Inhibition of XooJ2 growth by XooX1IDN and XooX2IDN *in vitro*

To evaluate the ability of phage XooX1IDN and XooX2IDN to lyse XooJ2 in liquid culture, a growth inhibition assay of host XooJ2 was performed as described under "Materials and Methods". The result showed that all XooJ2 cultures treated with phages (at all m.o.i) were less turbid compare to the XooJ2 culture without phages treatment (Fig. 6A). When XooJ2 cultures were initially

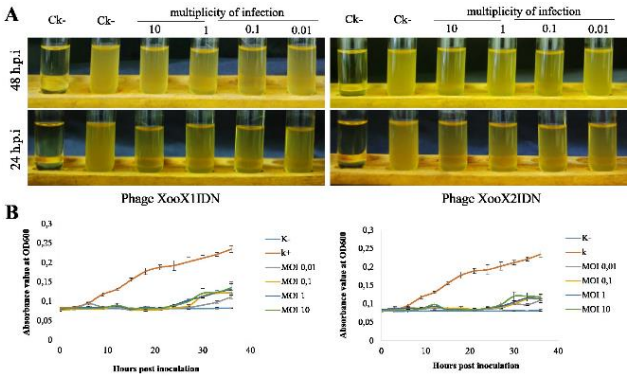

Fig. 6: Effects of phage XooX1IDN and XooX2IDN on the growth of XooJ2. (**A**) The XooJ2 growth characteristic in NB medium inoculated with phage XooX1IDN and XooX2IDN at different multiplicity of infection (m.o.i). Controls were medium with (Ck+) and without the host XooJ2 (Ck-). The culture turbidity was observed at 24 h (bottom) and 48 h (upper) after phage inoculation. (**B**) The growth curve of XooJ2 after inoculation with bacterial host and phage at m.o.i of 0 (red), 0.01 (green), 0.1 (purple), 1 (blue), and 10 (orange), respectively. The XooJ2 cell density was monitored by measuring the absorbance at 600 nm every 3 h for 36 h. The data are presented as the means from four replications for each m.o.i treatment. The error bars indicate the standard deviation

infected with phages (at all m.o.i) of both XooX11IDN and XooX2IDN, growth of the XooJ2 was inhibited until 24 to 27 h post-inoculation compared to control that the growth that was initially detected 6 h post-inoculation. Moreover, the growth of XooJ2 in liquid NB was significantly lower than in the cultures treated with the phage XooX1IDN and XooX2IDN. However, the turbidity of XooJ2 cultures treated with both phages at different m.o.i was not at significant level, compared to other m.o.is (Fig. 6B).

Discussion

Phages XooX1IDN and XooX2IDN are the first Xoo-infecting phages isolated from soil in rice field of Jember and Situbondo, Indonesia. Both bacteriophages were studied further, such as stability on temperatures, pH, plaque and particle morphology, host specificity, genome size, and structural protein profile. According to the transmission electron microscope examination, all phages have a phage morphology similar to phage having head and non-contractile tail (presented by short neck; Fig. 3). In addition, both phages, XooX1IDN and XooX2IDN are also possessed typical nucleic acid of myovirus that is double-stranded DNA with an average genome size of about 38–39 kb (Fig. 4). According to the morphology and nucleic acid type as mentioned on the guidelines of the International Committee on Taxonomy of Viruses (ICTV) (Ackermann 2003), all phages possess head and tailed particles may belong to the families of *Myoviridae*, *Siphoviridae*, or *Podoviridae* (Order *Caudovirales*). Moreover, phages

characterized by head and non-contractile tail (possesses short or long neck) commonly belong to the family of *Myoviridae*. The similar morphology and genome type were also reported to that myoviruses isolated from paddy field in China (Chae *et al*. 2014; Dong *et al*. 2018; Ogunyemi *et al*. 2019), phages isolated from tomato field in United State of America (Addy *et al*. 2019), or phage isolated from tomato in Japan (Fujiwara *et al*. 2008), which exhibited head and non-contractile tail phage particles.

The thermal stability of bacteriophages showed that the phage infectivity drastically decreased at the temperature of 60°C or more (Fig. 5A). Moreover, bacteriophages were completely lost their infectivity after incubation at a temperature of 80°C. Probably this condition may occur because the relationship of cross sulfide in the capsid protein of denatured bacteriophages at higher temperatures results in a loss of bacteriophage integrity (Jończyk *et al*. 2011). In the study, it was also revealed that all bacteriophages remained stable after treatment at various pH conditions, both in acidic and basic conditions, as the bacteriophage infectivity was still maintained even though it was treated at various pH levels (Fig. 5B). However, bacteriophages tend to be more stable in a pH range of 6 to 8. This phenomenon was also reported for the phage XOF4 that remained stable after growth at pH range of 6 to 8 (Ranjani *et al*. 2018). Temperature and pH contribute to the inactivation of bacteriophage particles by damaging their structural elements (Nobrega *et al*. 2016), phage aggregation, and ability to penetrate host cells (Langlet *et al*. 2007). On the other hand, phage XooX1IDN and XooX2IDN are the specific phages that infect only *X. oryzae*. This is typical phenomenon of bacteriophage and become the advantage of using phage as biological control agents since the phage only infect very narrow and specific bacterial strain (Dong *et al*. 2018; Elhalag *et al*. 2018; Ranjani *et al*. 2018).

The potency of phage XooX1IDN and XooX2ID to control XooJ2 was also tested to see how potent these two bacteriophages were, in suppressing the growth of the host XooJ2, qualitatively and quantitatively. The results demonstrated that XooX1IDN and XooX2IDN were able to control and inhibit the growth of *X. oryzae*. Although some cells showed steady growth phenomena, however, the cells growth remained significantly lower than control (Fig. 6), which indicates that equilibrium between lysis and cell growth was established or that phage-resistant cell growth rate might be decreased resulting the host population at a relatively low level. A similar result was previously reported for phage ΦRSL1 infecting *R. solanacearum* (Fujiwara *et al*. 2011), phage Xoo-sp2 infecting *X. oryzae* (Dong *et al*. 2018), or phage RsoM1USA infecting *R. solanacearum* (Addy *et al*. 2019).

Utilization and use of phage for biological control strategy have been widely reported as phage therapy against pathogenic bacteria (Fujiwara *et al*. 2011; Addy *et al*. 2012a; Elhalag *et al*. 2018). This phage therapy should contribute to enhancing the advantages of controlling bacterial leaf blight and reducing the use of conventional pesticides, which are harmful to the environment, human and animal health. Therefore, several steps must be examined during phage exploitation as biological control agent. All begins from the analysis of phage-host interaction *in vitro* followed by *in vivo* assay (Addy *et al*. 2012a). In this study, it is suggested that phage XooX1IDN and XooX2IDN have the potency to be used in controlling bacterial leaf disease. However, Dong *et al*. (2018) suggested that several studies must be done before utilize the phage for biocontrol to increase safety and sufficient implementation such as the host range, safety aspect of phage application, and mass production condition of phages. Therefore, some studies still needed to ensure that phage XooX1IDN and XooX2IDN are the best phages for phage therapy against bacterial leaf blight disease on rice, especially in Indonesia.

Conclusion

The XooX1IDN and XooX2IDN are the first *Xanthomonas oryzae* infecting bacteriophages that belongs to the family of *Myoviridae* and have a double-stranded DNA as genome with approximately about 39 kb and 38 kb in size. The bacteriophages remain stable by growth at maximum temperature of 60°C, indicating that these bacteriophages are suitable to use as biological control agent of bacterial leaf blight on rice.

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Author Contributions

All authors conceived and designed the research; DR performed the experiment; HSA and DR analysed the data and wrote the paper.

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