Lytic Bacteriophage for *Photobacterium damselae* Isolated from Water Environment

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Abstract—**The aims of this study were to isolate lytic bacteriophage of *Photobacterium damselae* and characterize it. Lytic bacteriophage of *Photobacterium damselae* was isolated from Ciparigi river in Bogor, Indonesia. Bacteriophages were enumerated by the double layer agar method. All bacteriophages isolate were morphologically and size plaque identified. Transmission electron microscope observation showed that the lytic bacteriophage of *Photobacterium damselae* (F A9) has an isometric head (30 nm in diameter) and contractrile tail (60 nm in length). This bacteriophage was grouped to *Myoviridae*. Protein analysis using SDS-PAGE showed four bands in poliacrilamide gel electrophoresis. Molecular weight of proteins F A9 were 14.96, 17.08, 30.58, and 109.06 kDa respectively.**

Keywords**: lytic bacteriophage, *Photobacterium damselae,* SDS-PAGE.**

I. INTRODUCTION

The source of infection diseases caused by bacterial could come from water, soil, food, or animals. *Photobacterium damselae* was bacteria that contaminate water and food. *Photobacterium damselae* could devide into two subspecies, *piscidia* and *damselae*. *Photobacterium damselae* subsp *piscidia* pathogen just to fish wheares *Photobacterium damselae* subsp *damselae* (formely *Vibrio damsela*) could pathogen not only to fish and mammals but also humans. Human infections could caused by bacteria pathogen that transmitted from fish or aquatic environment, direct contact with fish, or the infection source may be fish kept either for food [1].

Photobacterium damselae cause necrotizing fasciitis and septichemia that could be causing death. In 2002 reported a total of 18 patients, 13 patients from North America, 3 from Australia, 2 from Hong Kong infected with *Photobacterium damselae* after handling fish [2]. Surgically debride and amputate recommend to save the lives of patients [3]. Previously study report that toxin production of *Photobacterium damselae* correlation the ability of the bacterium to cause disease in mice (in vitro) [4]. These toxins also known could be spread in water [5]. *Photobacterium damselae* was known produce damselin toxins that cause hemolysis. Its gene (*dly)* was cloned and sequenced by [6], but further studies showed *dly* was not the only hemolysin in the sub species [7].

Photobacterium damselae could reduce using antibiotic, but antibiotic isn't recommended, actually in food and also there was resistance bacteria to antibiotic. *Photobacterium damselae* already resistant to several antibiotics including, ampicillin, amoxicillin and klavulanic acid, ciprofloxacin, clindamycin, amycasin, kanamycin, torbamisin, cefotaxime, erythromicin and tetracycline [8]. In order we need an alternative method, such as lytic bacteriophages. Lytic activity has been studied in an effort to overcome the diseases caused by bacteria [9].

Lytic bacteriophages can be used as a biocontrol and therapy. Lytic bacteriophages as a natural and non-toxic biological agent to reduce and control the growth of pathogenic bacteria because of its ability to lyse. Results of several studies have reported the effectivity of lytic bacteriophage lysis, example lytic bacteriophage *Shigella* FY51-X [10], *Shigella sonnei* SP18 [11], *Salmonella* sp FR 38, FR 19, and FR 84 [12], and lytic bacteriophage AB1 *of Acinetobacter baumanni* [13]. The diversity of bacteriophage host specificity makes it can be applied to aquaculture, humans, agriculture, veterinary, and food [14]. However there were no reports about lytic bacteriophage for *Photobacterium damselae* previously include in Indonesia. Therefore, this study was conducted to obatined a lytic bacteriophage *Photobacterium damselae* and knowing its characterization.

II. MATERIALS AND METHODS

Identification host bacterium

Host bacterium was previously isolated from faeces of diarhea [15]. The bacterium grown on triple soy agar (TSA) slant tube and incubated at 37 ^{0}C for 24 to 48 hours. Identification was done through gram staining and continued with API 20E. Species of host bacteria was identified therefore was done pathogenity test and observed by hemolytic activity on blood agar.

Isolation bacteriophage

Bacteriophage was isolated from Ciparigi river in Bogor, West Java of Indonesia. Samples were stored in sterile bottle. 4.5 mL of each water sample was added to 0.5 mL of host *Photobacterium damselae* broth cultures for enrichment and incubated overnight at 37 °C. The enrichment was subjected to low speed centrifugation at 2000 rpm for 20 minutes [16] with modification. The supernatant filtered through 0.22 µm pore size syringe filter. The filtrates were tested for the presence of bacteriophage.

Plaque Assay

Plaque assay was enumared using double layer agar (DLA) method by [17]. Bacteriophage samples diluting with *saline magnesium* (SM) buffer. Dilution of bacteriophage samples starting from 10^{-1} to 10^{-8}. Three of last dilution, each 200 µL bacteriophage samples were taken and added to 400 mL of the host culture, then incubated in heating bath at 37 ^{0}C for 30 min. The mixture was added to 5 mL of soft agar temperature of 47 ^{0}C for the top layer, then plating on the hard agar as bottom layer. Incubated at 37 ^{0}C for 24 hours. Observed plaques were formed [18].

Purification of Bacteriophage

Single plaque with its own characteristics derived from the plaque assay was transferred by Pasteur pipette into a tube, then mixed with 2-3 ml of SM buffer. The suspension is homogenized and allowed for 5-10 minutes at room temperature. The suspension was then centrifuged at 5000 rpm for 20 min and repeated twice. The supernatant was filtered using 0.22 µm pore size syringe filter to further stored as bacteriophage stock [19].

Determination of Host Range Bacteriophage

Determination of host range bacteriophage suspension is done by testing the bacteriophage with the bacterium *Photobacterium damselae* with different types of bacteria *(Salmonella* 38, 84 *Salmonella, Escherichia coli* Haemoragic (EPEC K1.1.), *Proteus mirabilis,* and *Bacillus pumilus.* Determination of host range tests conducted by modification techniques that the double layers agar of [18]. Positive response was indicated by the formation of plaque.

Bacteriophage morphology observation by Transmission Electron Microscope (TEM)

Bacteriophages dropped as much as 10 µL on a grid (400 mesh), wait for 20 seconds, then dried with filter paper. Five mililitre 2% uranyl acetate dripped into the top grid for 1 minute. Grid dried using filter paper and allowed to ± 20 minutes in order to completely dry. EM grids placed on the *holder*, allowed to dry. Once dried specimens examined by using a transmission electron microscope JEOL JEM-1010 models are operated 80kV at a magnification of 10000x - 100000x [20].

Analysis molecular weight protein of bacteriophage

The molecular weight of bacteriophage proteins were analyzed by *Sodium Sulphate-Poly Acrilamide gel electrophoresis* (SDS-PAGE) [21]. Marker used is *PageReguler™ Prestained Protein Ladder* with sequential molecular weights are respectively 10, 17, 28, 34, 48, 55, 72, 95, 130, and 180 kDa. Separator gel concentration of 12% polyacrylamide were placed on the bottom. The concentration of the gel was 7.5% polyacrylamide collector placed at the top of the gel after separation has become completely solid. Stock bacteriophage and the *molecular weight markers*, respectively mixed with the sample buffer at a ratio of 4:1 (4 parts sample and 1 part sample buffer). The mixture was homogenized, and then incubated for 24 hours. After that, heated in boiling water for 5 minutes, put in the gel wells with 60 µL volume. Electrophoresis was carried out with a current of 20 mA and a voltage of 50 volts for 3.5 hours. Electrophoresis was terminated when the dye samples reached the limit of 0.5 cm to 1 cm from the bottom of the gel. After ending electrophoresis, the gel removed from the glass plates and carried silver.

III. RESULTS AND DISCUSSION

Identification host bacterium

Gram staining showed that host bacterium was gram negative bacteria. First step to identified was known morphology cell of host bacteria. These could known from staining its cell through gram staining [22]. Based on physiologis test in API 20E (Table 1) 98,7 % was closely related to *Photobacterium damselae*. On blood agar, this bacteria has α hemolisis that could lyses a half of red cell. Identification of host for bacteriophage is very important. These was determine in sampling, as know as bacteriophage lived in host bacteria environment.

TABLE 1. CHARACTERIZATION PHYSIOLOGIS OF *Photobacterium damselae*

Characterization physiologis	Reaction	Characterization physiologis	Reaction
ONPG (Ortho NitroPhenyl- βD-Galactopiranosidase)	-	GLU (Glucosa)	+
ADH (Argine dihidrolase)	+	MAN (Manitol)	-
LDC (Lysine Decarboxylase)	-	INO(Inositol)	-
ODC (Ornithine Decarboxylase)	-	SOR (Sorbitol)	-

CIT (Citrate utilization)	-	RHA (Rhamnose)	-
H_2S (H_2S production)	-	SAC (Saccarose)	+
UREA (Urease)	-	MEL (Melibiose)	-
TDA (Trytophane Deaminase)	-	AMY (Amygdalin)	-
INDOL (Indol production)	-	ARA (Arabinose)	-
VP (Voges Proskaeur)	-	OKSIDASE	+
GEL (Gelatinase)	-	KATALASE	+

+ : positive reaction; - : negative reaction

Isolation Bacteriophage

Lytic bacteriophage of *Photobacterium damselae* was isolated from Ciparigi river Bogor, West java of Indonesia. Bacteriophage was signed by presence plaque on plaque assay. The presence of plaque on plate indicated lytic bacteriophage (Fig 1). Plaque could observed after incubation at 37 ^{0}C averange 18 until 24 hours. Plaque is cell of bacteria lysed by virus. One plaque indicated one cell bacteria lysed.

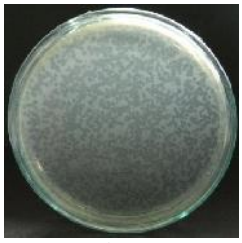

Fig. 1 Plaque forming on plate

Purification bacteriophage based on morphology and size of plaque. There were two lytic bacteriophage isolate. They are F $A9_1$ with concentration plaque forming unit 11 x 10^5 PFU/mL and F $A9_2$ 62 x 10^6 PFU/mL). F $A9_1$ and F $A9_2$ have clear and turbid plaque (Table 2). These plaque showed lytic bacteriophages, but both of plaque did not different bacteriophage. The evidence with purfication five times. In conclusion F $A9_1$ and F $A9_2$ are same bacteriophage isolate (F A9). Reference [23] reported that could occur conversion between clear and turbid plaque. Filamentous bacteriophage plaque could change if used high frequency of titer bacteriophage. There was many factor that could effect plaque forming. Time of lyse one of factor that determine morphology and size of plaque so that could appearing morphology plaque with different size [24].

TABEL 2. CHARACTERISTIC PLAQUE AND CONCENTRATION (PFU/mL) LYTIC BACTERIOPHAGE *Photobacterium damselae* (F A9)

Sample location	**Bacteriophage**	**Characteristic plaque**		**Concentration (PFU/mL)**
		Morphology plaque	**Diameter plaque (mm)**	
Ciparigi river	F $A9_1$	*clear* plaque	3	11 x 10^5
	F $A9_2$	*turbid* plaque	1	62 x 10^6

Host Range

Host range in this research (Table 3) showed F A9 spesific to *Photobacterium damselae* as its host. There was plaque forming on plates when F A9 added its host, but not when F A9 were added not its host (*Salmonella* 38, *Salmonella* 84, EPEC K 1.1, *Proteus mirabilis*, and *Bacillus pumilus*). Host range was related with suitability bacteriophage receptor. The proteins of bacteriophage as the receptor for attachment. Reseptor bacteriophage determine in first infection [25]. Specialized proteins called tail spikes and tail fibers of tailed bacteriophages. These proteins attach to specific structures on the outer surface of host bacteria. Flagellin, teihoic acids, capsular polysaccharides (K-antigens), lipopolysaccharides (O-antigens) and also specific membrane proteins can be used as attachement sites [26]. Reference [27] assumed that the primary and most component of determining host range was interaction between the bacteriophage's host interaction protein and cell's receptor.

TABLE 3. HOST RANGE OF F A9

Bacteriophage	**Host bacteria**					
	***Salmonella* 38**	***Salmonella* 84**	**EPEC K.1.1**	***Proteus mirabilis***	***Photobacterium damselae***	***Bacillus pumilus***
F A9	-	-	-	-	+	-

+ : plaque; - : no plaque

Observation morphology of bacteriophage with Transmission Electron Microscope (TEM)

Morphology F A9 on TEM (JEOL JEM-1010 80kV) with 50,000x magnification showed F A9 has isometric head (30 nm in diameter) and contactrile tail (60 nm in lenght) (Fig 2). The size of head and tail of F A9 smaller than the other *myovridae* family have been reported. Based on its characteristic and also according to *International Committee on Taxonomy of Viruses* (ICTV) F A9 was family of *Myoviridae*. *Myoviridae* was family of bacteriophage that has an isometric head and contractile tail. Its member the second one mostly 1312 [28]. For this research I assumed that F A9 related to T4 bacteriophage which one of *Myoviridae* family too.

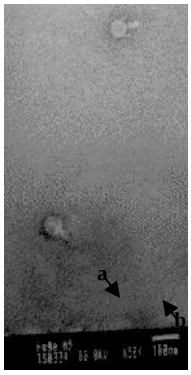

Fig. 2 Morphology of F A9 50000x magnification with 2% uranyl acetate; a) head (30 nm in diameter) and b) contractrile tail (60 nm in lenght), bar = 100 nm.

Molecular weight protein of F A9

In first well on poliacrylamide gel electrophoresis showed molecular wiight protein of marker and second well was control (aquabides). There are four bands protein of F A9 in third well. In first lane showed molecular weight protein was 109.06 kDa, lane 2 was 30.58 kDa, lane 3 was 17.01 kDa, and lane 4 was 14.96 kDa (Fig 3). These band indicate protein arrange bacteriophage which are proteins of head, tail, and enzyme lytic activity.

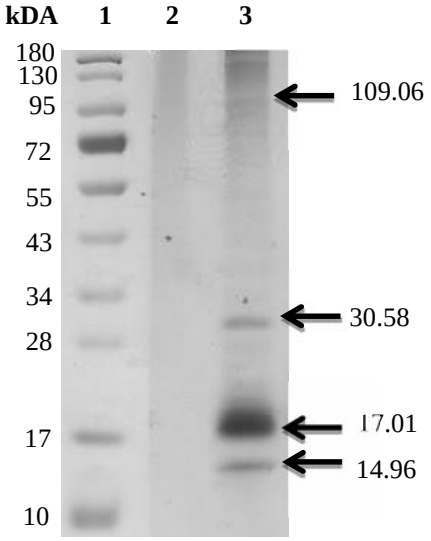

Fig. 3 Profile protein by SDS-PAGE analysis ; lane 1, Marker, lane 2, Control (Aquabides), lane 3, F A9

There is no report for molecular weight protein of lytic bacteriophage for *Phtobacterium damselae* previously. The proteins of F A9 could be part of capsid, tail, or endolysin (lytic enzyme). [29 reported that bacteriophage for *Thermus thermophillus* which is family of *Myoviridae* have molecular weight protein were 31 kDa and 66 kDa. *Vibrio* bacteriophage reported that also grouped in *Myoviridae* have molecular weight protein were 29 kDa, 43 kDa, and 68 kDa [30]. Compared of molecular weight protein in this research, there was difference. It could be showed strain of *Myoviridae* that have different of profile protein.

IV. CONCLUSION

Lytic bacteriophage *Photobacterium damselae* (F A9) obtained from Ciparigi river Bogor, West Java, Indonesia. F A9 has isometric head and contractile tail. It could grouped to *Myoviridae*. Analysis protein of F A9 showed four bands that indicate molecular weight protein. These band could indicate protein of bacteriophage that could be structural and functional protein (Endolysin).

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