FORMULATION OF BACTERIOPHAGES FOR CONTROLLING BACTERIAL BLIGHT DISEASE CAUSED BY *Xanthomonas oryzae* pv. *oryzae* IN RICE

By

LIU JIAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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DEDICATION

This thesis is dedicated to All the People
who give me inspirations, support and encouragement

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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June 2021

Chairman : Tan Geok Hun, PhD
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Bacterial leaf blight (BLB) disease caused 80 % of disease incidence in paddy in Kedah and Selangor states of Malaysia. Bacteriophages (or phage) have been proposed as a technique of pathogen control due to their efficiency and safety compared to destructive chemicals. In this study, The *Xoo*-phage was isolated and characterized as the first objective. A total of 70 *Xoo*-phages were isolated from termite, which three lytic phages were selected due to high titre of the virus (approximately 10^8-10^9 pfu/ml), and those phages belonged to the family *Podoviridae*, order *Caudovirales* with short, non-contracted tails. In additional, these phages have narrow host range specifically target *Xanthomonas oryzae.* Moreover, the latent time for Nϕ-1, Nϕ-2 and Nϕ-3 were estimated to be about 10, 10, and 20 min, respectively, while the burst size of these phages was 3.4×10^5, 1.23×10^5, and 4.2×10^5 pfu/ml, respectively. However, phages are sensitive, and infection efficiency was dropped when exposing to harmful environments. Thus, the second objective was to evaluate the skim milk, rice flour, corn flour, and CalnuXan (calcium and magnesium) as a carrier to formulation the isolated phage to maintain the phages activity under extreme pH and temperature condition. All formulated phages retained their activity at pH 5, pH 7 and pH 9, However, pH 7 remained the optimal pH compared to other pH treatments, showing a higher titre of approximately 1-2 log10 pfu/ml by following 24 h exposure to 37 °C. Besides, the formulated phages also retain hightitre compared with unformulated phages by expose to high temperature (37 °C and 45 °C). Based on the *in vitro* study of formulation, the last objective was to investigate the efficacy of formulated bacteriophages in the glass house. while the plant height, leaf chlorophyll, disease scoring, and rice plant weight was evaluated. Formulated *Xoo* phages shown higher of rice plant height compared to untreated group at day 28, 35 and 42. In additional, the unformulated treatment shown higher chlorophyll content compared to those of the untreated control, but significantly lower chlorophyll content compared to those of the formulated control. Subsequently, the rice plant has relative low disease severity (below 30 %) for all thetreatment groups with or without formulation at

day 14 compared to those of the untreated rice plant that showed above 60 % severity. the present results demonstrated that skim milk, corn flour, and rice flour are potential carriers to protect the phage under unfavourable environments, including extreme pH andtemperature. It also increases the control efficacy of BLB disease in glass house. In summary, this study successfully characterized two novel *Xanthomonas* phages and their potential as anti-microbial agents to against BLB disease in rice.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

FORMULASI BAKTERIOFAJ MENGAWAL PENYAKIT HAWAR PADI YANG DISEBABKAN OLEH *Xanthomonas oryzae* pv. *oryzae* DI PADI

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Penyakit hawar padi (BLB) telah menyebabkan 80 % pencetusan penyakit padi di negeri Kedah dan Selangor Malaysia Bakteriofaj (atau faj) telah dicadangkan sebagai teknik mengawal patogen disebabkan keberkesanannya dan keselamatan penggunaan berbanding dengan penggunaan perosak kimia. Faj *Xoo* telah diisolat dan ciri-ciri kepastian sebagai objektif pertama. Sebanyak 70 isolat faj telah diisolat daripada anai-anai dan hanya tiga bakteriofaj lisis dipilih menunjukkan titer yang tinggi (kira – kira 10^8-10^9 pfu/ml) dan faj tersebut tergolong dalam order *Caudovirales* dengan pendek, ekor tidak berkontraksi dan klasifikasi dalam keluarga *Podoviridae.* Tambahan pula, faj ini mempunyai julat tuan rumah yang sempit secara khusus mensasarkan *Xanthomonas oryzae*. Di samping itu, masa pendam bagi Nϕ-1, Nϕ-2 dan Nϕ-3 kira-kira dalam 10, 10, dan 20 min, manakala saiz pecahan faj iaitu 3.4×10^5, 1.23×10^5, dan 4.2×10^5 pfu/ml. Walaubagaimanapun, faj sangat sensitif dan jangkitan efisien akan menurun apabila mendedahkan dalam persekitaran yang berbahaya. Oleh itu, objektif kedua dalam kajian ini adalah untuk menilai penggunaan susu skim, tepung beras, tepung jagung dan CalnuXan (kalsium dan magnesium) sebagai pembawa dalam formulasi isolat faj untuk melindungi faj daripada penukaran pH dan suhu yang tidak bersesuaian. Semua formulasi faj dikekalkan dalam aktiviti pada pH 5, pH 7 dan pH 9. Walaubagaimanapun, pH 7 kekal dalam optimal pH dibandingkan dengan pH rawatan lain menunjukkan titer yang tertinggi kira-kira 1-2 log10 pfu/ml berikuti 24 h terdedah dalam suhu 37 °C. Selain itu, formulasi faj turut kekal titer tertinggi berbanding dengan formulasi faj yang terdedah dalam suhu tinggi (37 °C dan 45 °C). Berdasarkan kajian formulasi *in vitro*, objektif terakhir adalah untuk mengaji keberkesanan formulasi bakteriofaj dalam rumak kaca, sementara ketinggian padi, lebar, klorofil daun, penyakit penilaian, dan berat padi turut dicatatkan. Padi yang dirawat daripada formulasi *Xoo* faj menunjukkan padi yang tertinggi berbanding dengan padi yang tidak dirawat pada hari 28, 35 dan 42. Tambahan pula, padi yang tidak dirawat menunjukkan kandungan klorofil yang tinggi berbanding dengan padi yang tidak dirawat (*untreated control*) tetapi

menunjukkan kandungan klorofil yang rendah berbanding dengan padi yang dirawat (*treated control*). Selain itu, padi mempunyai jangkitan penyakit yang rendah (bawah 30 %) untuk semua kumpulan padi yang dirawat dan tidak dirawat dalam formulasi faj pada hari 14 berbanding dengan padi yang tiada semburan formulasi faj yang menunjukkan lebih 60 % jangkitan penyakit. Berdasarkan keputusan pengajian menunjukkan susu skim, tepung jagung dan tepung beras berpotensisebagai pembawa untuk melindungi faj daripada penukaran pH dan suhu yang tidak menentu. Pembawa tersebut turut meningkatkan kawalan yang efisien terhadappenyakit BLB di rumah kaca. Kesimpulannya, pengajian ini telah berjaya isolat dua novel *Xanthomonas* faj dan berpotensi sebagai agen anti-mikrob untuk melawan penyakit BLB terhadap padi.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF SYMBOLS AND ABBREVIATIONS

-	minus
-	to
%	Percentage
~	About
+ve	Positive
<	Less than
=	Equal
µg	Microgram
µm	Micrometre
AGB	Above-ground-biomass
ANOVA	Analysis of variance
b	Billion
BCA	Biological control agentts
BLB	Bacterial leaf blight
BLS	Barat Laut Selangor
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
cfu	Colony forming unit
cm	Centimeter
CRD	Completely randomized design
DNA	Deoxyribonucleic Acid
ds	Double-strand
e.g	Example
EPS	Extracellular polysaccharide slime
FAO	Food and Agriculture Organization
Fe^{2+}	Ferrous ion

g	Gram
GDP	Gross Domestic Product
h	Hour
H_2SO_4	Sulfuric Acid
ha	Hectares
IDM	Integrated management of diseases
IRRI	International Rice Research Institute
KADA	Kemubu Agricultural Development Authority
kb	Kilobase
KOH	Potassium hydroxide solubility
LAI	Leaf area index
LB	Luria Bertani
M	Molar
m	meter
M ha	Million hectares
MADA	Muda Agricultural Development Authority
MARDI	Malaysian Agricultural Research and Development Institute
Mg^{2+}	Magnesium ion
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
min	Minute
mL	Millilitre
mM	Millimolar
mm	Millimetre
MMT	Million metric tonnes
MOI	Multiplicity of infection
MT	Million tonnes
NA	Nutrient agar
NaCl	Sodium chloride
nm	Nanometers

NPK	Nitrogen, Phosphorus, and Potassium
NYA	Nutrient agar yeast extract medium
O.	Oryza
^{o}C	Degree Celcius
OD	Optical density
ϕ	Phage/Bacteriophage
PDI	Per cent indices
PEG	Polyethylene glycol
pfu	Plaque forming unit
PGPR	Plant growth-promoting rhizobacteria
pH	Potential of hydrogen
PPM	Parts Per Million
PROC	Per cent reduction over control
PSA	Peptone sucrose agar
pv	Pathovar
RFES	Rice filed ecosystems
RM	Malaysian ringgit
RNA	Ribonucleic Acid
rpm	Revolutions per minute
Sp.	Species
spp	Species plural
T	Treatment
TEM	Transmission Electron Microscopy
UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B
v/v	Volume per volume
-ve	Negative
w/v	Weight per volume

WSSA	Wakimoto semi-synthetic agar
Xoo	*Xanthomonas oryzae* pv. *oryzae*
YDCA	Yeast extract dextrose calcium carbonate

CHAPTER 1

INTRODUCTION

1.1 Background

Rice *(Oryza sativa L.)* is the primary food grain consumed by almost half of the world's population (Onasanya *et al*., 2010). It plays a significant role in world food production, which is 90 % of production and consumption in Asian countries (Onasanya *et al*., 2010). FAO, 2015; Bandumula, 2018). The demand for rice increased every year due to the increase in the world population. It provides 40 % of the total calorie and supports 60 % of the population; over 3.5 billion people counton rice for their daily calory intakes (Cheng *et al.*, 2007; Herman *et al.,* 2015).

Rice is vulnerable to several diseases such as bacterial blight, blast and sheath blight (Dai *et al*., 2007). In September 2017 and April 2018, Azizi *et al.* (2019) reported that rice varieties (MR 269 and CL varieties) were infected with bacterial leaf blight disease and caused 80 % disease incidence in Kedah and Selangor states of Malaysia. The bacterial leaf blight (BLB) disease in the rice fields was reported in Peninsular Malaysia (Saad, 1995). The disease mainly generated by the infection of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), particularly for the lowland irrigated and rainfed rice in Asia's tropical and temperate environments (Gumma *et al.,* 2011). It is quickly developed when the temperatures fall at 28-34 °C with above 70 % relative humidity (Busungu *et al*., 2016; Chukwu *et al*., 2019). Infection of plants bythe BLB disease at maximum tillering stage causes in 20–50 % reduction in the harvest which tends to get serious at a preliminary stage that accounts for 50 % loss of crops (Zhang and wang, 2013; Yasmin *et al.,* 2017).

The genus of *Xanthomonas* is classified into Gram-negative bacterial and potential to infect at least 350 different plants (Chatterjee and Sonti, 2002; Zeriouh *et al.,* 2011; Qian *et al.,* 2013). At present, Chukwu *et al.* (2019) have reported that BLB disease affected the whole rice growing area because there are no variety of rice species to resistant BLB disease. Many approaches have been evaluated in order to manage the incidence of disease in rice planting areas, which including chemical control, host resistance and cultural control (Laha *et al.,* 2017). Currently, the addition of bactericides, streptomycin, chloramphenicol and niclosamide are broad used to control this disease (Khan *et al*., 2012; Chen *et al*., 2015; Kim *et al*., 2016). However, the BLB disease's chemical control is defective due to the inability to obtain suitable bactericides to inhibit the development of the disease and its effects (Saad and Habibuddin, 2010). Besides, chemical pesticides also caused a negative impact on the environment. Therefore, efficient and pollution-free approaches were applied to controlling and prevent the BLB incidence in rice production (Schantz *et al*., 2001).

Therefore, some alternative approaches were applied to control and prevent the BLBincidence in rice production. Biological control agent (BCA) could be an alternative method to manage the plant diseases resulted from the infection of caused by these pathogenic bacteria. Specific plant growth-promoting bacteria, such as *Pseudomonas*, *Bacillus* spp. (Udayashankar *et al*., 2011; Pérez -Montaño *et al.,* 2014;Yasmin *et al.,* 2017) and *Pseudomonas aeruginosa* BRp3 (Yasmin *et al.,* 2017) were used as "biological control agents" (BCA) on rice.

Bacteriophage (Phage), a virus that infects explicitly bacteria, could also be used to be efficient biological agents. Phage therapy is alternative approaches were applied active phage to an inhibition plant disease caused by bacteria (Bae *et al.,* 2012). Phages have been reported to control several bacterial diseases in the plant (Balogh *et al*., 2010), including canker disease caused by *Xanthomonas citri subsp. citri* in citrus (Ibrahim *et al*., 2017). Besides that, Yin *et al.* (2019) also reported that the bacteriophages cocktail application has the potential to control bacterial canker in kiwifruit caused by *Pseudomonas syringae* pv. *actinidiae*. In addition, bacteriophage therapy effectively combats soft rot diseases and bacterial wilt on potatoes caused by *Dickeya solani* and *Ralstonia solanacearum*, respectively (Adriaenssens *et al.,* 2012; Wei *et al.,* 2017). Moreover, Dong *et al.* (2018) have reported that *Xoo*-sp2 phage has been used to control the BLB disease. Following, the characterize of *Xoo*-sp2 has been done. However, there is no report on the formulation of *Xoo* phages to expose at different temperatures and various pH values to check the stability under extreme conditions. Therefore, this study evaluates the potential of isolated phages formulated with different carriers under extreme conditions.

1.2 Problem Statement

However, one of the challenges of using bacteriophages as BCA is that it has a shorttime to reside on plant foliage due to harmful environmental factors such as rain andsunlight-UV. Thus, the formulation of bacteriophages is needed to increase the bacteriophages longevity on the plant surface (Balogh *et al.*, 2003). Appropriate time of application is also essential to maintain the survival of bacteriophages on plant foliage, leading to the increased efficacy of bacteriophages treatment for the plant disease (Jones *et al.*, 2012). The researchers' reports indicate that the bacteriophage formulated with milk powder compositions, plant peptone, and soy protein could be recovered and actively maintained. Balogh *et al.* (2003) also showed that bacteriophage formulated with skimmed milk and casein could significantly reduce plant diseases and reduce plant diseases by 79 % compared to that of the unformulated bacteriophage. Based on the previous researcher, the unformulated phage's titre was decreased faster at room temperature after 36 h and 48 h. However, the formulated phages reduce the titre to only two days (Abo-elmaaty *et al*., 2016). In addition, skim milk, cornflour, and sucrose are carriers to protect the phage under extreme conditions (Iriarte *et al.,* 2007; Tewfike and Desoky, 2015; Orynbayev *et al.,* 2020). Recently, Born *et al*. (2015) reported that the other carriers had been investigated for phage protection. Such as nature compounds (juice of beetroot, red pepper and carrot). However, this no report on cornflour and Calnuxan as carriers to protect phage. In this present study, the need

arose for new carriers to formulation phages and protected them.

Development of phage stability and survival rate through formulated with different carriers in vitro study is necessary. However, the formulated phage was applied to the glasshouse trail to investigate the disease severity's reduced rate against the BLBdisease. Chae *et al.* (2014) mentioned that PM and PMh phage were diluted in skim milk (0.75g/L) that showed potential against BLB disease and decreased the BLB infected leaf area under the field trial. The same parameter was measured for formulated phage of this research. In contrast, this research's objective was to observe the disease severity every 7 days till 42 days after treatment. In addition, the plant height and chlorophyll content were also measured to evaluate the formulated phage under the glasshouse under extreme conditions.

1.3 Research Objective

This study hypothesised that different carriers successfully formulated the isolated phage, protected it under extreme conditions, and kept high phage titre. In addition, formulated bacteriophage specifically targets *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) could reduce the BLB disease incidence and improve rice yield, improve the plant height and chlorophyll content.

The objective of this study is to develop a formulated bacteriophage to control *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Specific objectives of the study as follows:

1. To isolate and characterise potential bacteriophages to control *Xanthomonas oryzae* pv. *oryzae* (*Xoo*)

2. To develop bacteriophages formulation using different carriers

3. To evaluate the effect of bacteriophages on disease severity in the glasshouse

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