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CHARACTERISATION OF BACTERIOPHAGES FOR CONTROLLING BACTERIAL BLIGHT DISEASE IN RICE

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By

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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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"So, verily, with every difficulty there is relief,
Verily, with every difficulty there is relief,
Therefore, when thou art free
(from thine immediate task), still labor hard,"
[Al-Insyirah: 5-7]

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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Chairman : Tan Geok Hun, PhD
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Rice cultivation is the principle activity and source of income for millions of household around the globe and several countries in Asia and Africa. Over 90 percent of the world's rice is produced and consumed in the Asia-Pacific Region. Bacterial leaf blight (BLB) disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the most serious disease of rice in South-East Asia. There are many approaches in order to combat the disease. Chemical application and antibiotics are among the most famous and easy method used to suppress the disease. However, the long-term usage may render harmful side effects to the ecosystems and may lead to the emergence of resistant-bacteria strains. Thus, the present study was carried out with the aim to isolate bacteriophages from various sources for controlling local *Xoo* in rice. The second aim would be to isolate and identify *Xoo* from infected rice plant which served as a host for isolation of potential phages. The bacteriophage isolates were analysed based on their lytic activity towards *Xoo*, host range, electron microscopy and their genomes were isolated to study their type, size and restriction enzyme profile patterns. The structural proteins of the phages were also studied by SDS-PAGE. Subsequently, the efficacy of a selected phage to reduce the incidence of BLB which *Xoo* as a host bacteria load in glass house was evaluated. Six lytic bacteriophages namely Qϕ-146, Qϕ-156, Qϕ-158, Qϕ-160, Qϕ-161 and Qϕ-162 were isolated from infected rice soil samples. Phage Qϕ-161 was found to demonstrate a broader host range in which it was able to infect *Xanthomonas campestris, Ralstonia solanacearum, Bacillus methylotropicus* and *Bacillus siamensis* apart from *Xoo* (the original host). The morphology of these phages indicated that they belonged to the *Podoviridae* family. All six phages revealed similar protein profile when analysed using SDS-PAGE with three major bands of proteins which had molecular weight of 100, 36 and 15 kDA. All phages had genome size of approximately 23 kb and differentiated by their *EcoRI, BamHI* dan *HindIII* restriction fragment patterns. Based on the overall characteristics of the phage isolates, all six phage isolates were then mixed to form phage cocktails for subsequent glass house study. With approximate 10^8 pfu/ml titer application

of phage cocktails in rice challenged with approximate 10^8 cfu/ml of *Xoo*, it was found to reduce the incidence of BLB in rice. Untreated rice plant, exhibited severe infection symptoms on the leaves with a disease severity rate of 46.67% compared to treated plant, T1 with 23.61% on 42-day post-treatment. In conclusion, phages demonstrated potential as an alternative control method to increase the control efficacy of BLB and reduce the use of agrochemicals. However, further characterisation on phage-host interaction such as the effects of different pH, temperature and multiplicity of infection (MOI) ratio should be carried out. Further optimisation of both dosage and alternative routes of phage administration should also be carried out to enhance the efficacy of the phage.

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

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Penanaman padi adalah aktiviti asas dan punca pendapatan bagi berjuta-juta isi rumah di serata dunia dan di beberapa negara Asia dan Afrika. Lebih 90 peratus padi di dunia ini dihasilkan dan digunakan dalam rantau Asia Pasifik. Penyakit hawar padi (BLB) yang disebabkan oleh *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) adalah penyakit padi yang paling serius berlaku di Asia Tenggara. Terdapat pelbagai pendekatan untuk menangani penyakit ini. Penggunaan bahan kimia dan antibiotik adalah antara kaedah yang paling terkenal dan mudah digunakan untuk menyekat penyakit ini. Walau bagaimanapun, penggunaan dalam jangka masa yang panjang akan memberikan kesan sampingan yang boleh membahayakan ekosistem dan boleh menyumbang kepada kemunculan strain bakteria yang rentan. Tujuan kedua adalah untuk mengasingkan bakteriofaj dari pelbagai sumber untuk mengawal bakteria tempatan *Xoo* dalam padi. Selain itu, bagi mengasing dan mengenal pasti *Xoo* dari pokok padi yang dijangkiti untuk digunakan sebagai sel perumah bagi mengasingkan faj yang berpotensi. Isolat bakteriofaj dianalisis berdasarkan aktiviti lisis terhadap *Xoo,* julat perumah, mikroskopi electron dan pemencilan genom untuk mengidentifikasi jenis, saiz dan juga pola profil genom menggunakan enzim penyekatan. Struktur protein faj juga telah dikaji menggunakan SDS-PAGE. Kemudian, keberkesanan faj dalam mengurangkan kejadian penyakit BLB terhadap bakteria perumah, *Xoo* dijalankan di rumah kaca. Enam bakteriofaj lisis iaitu Qϕ-146, Qϕ-156, Qϕ-158, Qϕ-160, Qϕ-161 dan Qϕ-162 yang telah diasingkan daripada sampel tanah padi yang dijangkiti telah dipilih. Faj Qϕ-161 didapati menunjukkan julat perumah yang lebih luas di mana ia mampu menjangkiti *Xanthomonas campestris, Ralstonia solanacearum, Bacillus methylotropicus* dan *Bacillus siamensis* selain *Xoo* yang merupakan perumah asal di mana ia dipencil. Morfologi faj ini menunjukkan bahawa mereka tergolong dalam keluarga *Podoviridae*. Keenam-enam faj menunjukkan profil protein yang sama apabila dianalisis menggunakan SDS-PAGE dengan tiga lapisan utama protein yang mempunyai berat molekul diantara 100, 36 dan 15 kDa. Kesemua faj mempunyai saiz genom kira-kira 23 kb dan telah dibezakan menggunakan pola serpihan penyekatan *EcoRI, BamHI* dan *HindIII*. Berdasarkan ciri-ciri keseluruhan

faj yang diasingkan, kesemua enam faj kemudiannya telah dicampur untuk menghasilkan koktel faj untuk kajian berikutnya di rumah kaca. Dengan penggunaan kira-kira 10^8 pfu/ml titer koktel faj yang diaplikasi ke atas pokok padi yang kemudiannya dicabar dengan anggaran titer *Xoo* sebanyak 10^8 cfu/ml, dan didapati ianya dapat mengurangkan insiden BLB pada pokok padi. Berbeza dengan pokok padi yang tidak dirawat, semua pokok memberikan simptom jangkitan yang teruk dan menunjukkan gejala yang teruk pada daun dengan peratusan jangkitan sebanyak 46.67% berbanding dengan T1 yang dirawat dengan peratusan jangkitan sebanyak 23.61% pada hari ke-42 selepas rawatan. Kesimpulannya, faj berpotensi digunakan sebagai satu kaedah alternatif kawalan untuk meningkatkan keberkesanan kawalan terhadap penyakit BLB dan mengurangkan penggunaan agrokimia. Walau bagaimanapun, pencirian tambahan pada interaksi antara faj-perumah seperti kesan terhadap pH, suhu dan kajian nisbah gandaan jangkitan (MOI) yang berlainan perlu dijalankan. Pengoptimuman tambahan pada dos dan laluan alternatif untuk aplikasi faj perlu dijalankan bagi meningkatkan keberkesanan faj tersebut.

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LIST OF SYMBOLS AND ABBREVIATIONS

$NH_4H_2PO_4$	Ammonium dihydrogen phosphate
ANOVA	Analysis of Variance
bp	Base pair
β	Beta
BCP	Bromcresol purple
BTB	Bromothymol blue
BLB	Bacterial leaf blight
BLB-2	Isolates of *Xanthomonas oryzae* pv. *oryzae* from pathogenicity test
BUG	Blood universal growth
BSA	Bovine serum albumin
cm	Centimeter
CTAB	Cetrimonium bromide
CABI	Centre for Agriculture and Biosciences International
CLSI	Clinical Laboratory and Standard Institute
CBB	Coomassie Briliant Blue
cfu	Colony forming unit
Na_2HPO_4	Disodium hydrogen phosphate
d H_2O	Distilled water
^oC	degree Celcius
dNTP	Deoxynucleotide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DS	Disease severity
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPS	Extracellular polysaccharide slime
EPPO	European and Mediterranean Plant Protection Organization
FAO	Food and Agriculture Organization

FCI	Food Corporation of India
g	Gram
HSD	High Significant Difference
H_2O_2	Hydrogen peroxide
IRRI	International Rice Research Institute
ICTV	International Committee on Taxonomy of Viruses
kbp	Kilobase pair
kDa	Kilodalton
MADA	Lembaga Kemajuan Pertanian Muda
<	less than
≤	less or equal than
L	Liter
LB	Luria-Bertani
$MgCl_2$	Magnesium chloride
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
MARDI	Malaysian Agricultural Research and Development Institute
µl	Microliter
µg	Microgram
mg	Milligram
ml	Milliliter
mM	Millimolar
mm	Millimeter
mV	Millivolts
M	Molar
>	more than
≥	more or equal than
NCBI	National Center for Biotechnology Information
-ve	Negative
NA	Nutrient agar
NB	Nutrient broth

nm	Nanometer
NYA	Nutrient agar yeast extract medium
NPK	Nitrophoska Green
OD	Optical density
pv	Pathovar
%	Percentage
ϕ	Phage / bacteriophage
PBS	Phosphate buffer saline
pmol	Picomole
pfu	Plaque forming unit
+ve	Positive
KCl	Potassium chloride
KOH	Potassium hydroxide
PEG	Polyethylene glycol
PCR	Polymerase Chain Reaction
K_2HPO_4	Potassium hydrogen phosphate
PSA	Peptone sucrose agar
RCBD	Randomized complete block design
rpm	Revolutions per minute
RNA	Ribonucleic Acid
16S rRNA	16s Ribosomal Ribonucleic Acid
ASM	Acibenzolar-S-methyl
NaCl	Sodium chloride
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sp.	Species
SD	Standard deviation
SAR	Systemic acquired resistance
TBE	Tris-borate-EDTA
TE	Tris-EDTA

TEMED	Tetramethylethylenediamine
TEM	Transmission Electron Microscopy
USA	United States of America
UK	United Kingdom
UV	Ultraviolet
Xoo	*Xanthomonas oryzae* pv. *oryzae*
XOR	*Xoo* isolates from infected rice leaves with BLB
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Rice (*Oryza sativa*) is the staple food for more than half of world's population. Rice provided 19% of global human per capita energy and 13% of per capita protein in 2009, where more than 3.5 billion people (more than 20%) depended on rice for their daily calories. Asia accounts for 90% of global rice consumption and the total rice demand continues to rise (GRiSP, 2013). However, in South-East Asia and most of the rice growing countries, *Xanthomonas oryzae* pv. *oryzae (Xoo),* a plant pathogen that causes bacterial leaf blight (BLB) disease which is the most destructive rice disease resulting in a significant yield reduction of rice production under serious infestation (Mew *et al.,* 2004). In early 80s, the incidence of BLB was perceived in the Peninsular Malaysia rice fields and the estimated loss was about RM 50 million during 1982 to 1994 (Saad and Habibuddin, 2010). The recent outbreak of this disease was described occur at Padang Besar, Perlis with only 60,000 metric tones of rice production in February 2014 (Utusan, 2014).

There are different approaches that have been used to reduce the incidence of the disease, such as using seed treatments (Singh and Monga, 1985), soaking of rice seedlings in antibiotic at transplanting (Durgapal, 1983), host plant resistance to the disease (Ramalingan *et al.,* 2003; Mew *et al.,* 2004) and bacterisation of seeds with *fluorescent Pseudomonads* (Anuratha and Gnanamanickam, 1987). The recent approach using *Bacillus* plant growth-promoting rhizobacteria spp., which is an antagonist to *Xathomonas oryzae* (Chithrashree *et al.,* 2011; Wu *et al.,* 2015), has been tried as a method of biological control to combat the disease. However, none of these methods were effective in controlling the disease in the long run due to the emergence of bacterial resistance to these agents.

Phage therapy has been proven effective against several types of bacterial diseases due to the specificity character of the virus (Jones *et al.,* 2007; Balogh *et al.,* 2009). Phages are viruses that infect bacteria, which multiply in a specific bacterial host and eventually lyse them. They are ubiquitous in nature and can be divided into two types which are obligate lytic and temperate phages (Gill and Hyman, 2010). For phage therapy purpose, which is the use of phages to kill bacteria, only lytic phages are employed. Since these phages are specific against the bacterial host, they have a great potential to be used as biological control agent against certain plant pathogens.

Phage therapy has been shown as an attractive candidate to cope with persistent pathogen infections (McVay *et al.,* 2007). Phage therapy or biocontrol agents of pathogen using phages have been used for more than a century. Felix d'Herelle first discovered bacteriophage in the early 1900s, where phages were initially screened as promising agents to control diseases in the veterinary medicine field (Maxson-Stein *et al.,* 2002;

Summers, 2004). In betimes, phage therapy study was restricted due to insufficient facts available on phages at that time. Research was conducted decades later on temperate and virulent phages. The appropriate purification methods and phage host range were also conducted effectively for clinical testing.

Phage control has been analysed on poultry (Atterbury *et al.,* 2007) and compost (Heringa *et al.,* 2007) with some demonstrated success in controlling pathogens in plants. These include control of phytopathogens on tomato bacterial spot (Obradovic *et al.,* 2004; Obradovic *et al.,* 2005), potato soft rot (Czajkowski *et al.,* 2014a; Czajkowski *et al.,* 2015), citrus bacterial canker (Balogh *et al.,* 2008), apple and pear fire blight (Boulé *et al.,* 2011), geranium bacterial blight (Flaherty *et al.,* 2001), onion bacterial blight (Lang *et al.,* 2007) and mushroom bacterial blotch (Kim *et al.,* 2011).

The production of phages predominantly required a combination of host growth and subsequent purification (Gill and Hyman, 2010). Enhancement in technology of phage purification reduces the cost, while the cost of host growth relies on the bacterial species (Kramberger *et al.,* 2010). Generally these costs of phage production, per unit (Kutter *et al.,* 2010) are commensurate with the costs of pharmaceutical production while the costs of isolation (discovery) and characterisation can be quite low (Skurnik *et al.,* 2007).

The use of the natural alternative to reduce the usage of chemicals can be solved by using phages and implemented as one of the integrated management strategies for most of the plant disease. They are excellent candidates for extensive use in developing countries due to the low production cost and simple preparation of phage treatments. By far, phages have not been reported to be toxic. Therefore, in this study it is hypothesised that phage may able to be utilised as biocontrol agent for BLB in rice.

1.1 Objectives of the Study

This study was conducted with three objectives;

1.1.1 To isolate, characterise and identify *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) from the infected rice.

1.1.2 To isolate, characterise and screen the potential bacteriophages for controlling *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in the rice.

1.1.3 To evaluate the efficacy of bacteriophages against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) as a biological control agent in the glass house.

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