



UNIVERSITI PUTRA MALAYSIA

***CHARACTERIZATION OF STREPTOMYCES AMBOFACIENS S2 AND
ELUCIDATION OF ITS ANTI-FUNGAL COMPOUNDS FOR BIOCONTROL
OF CHILLI ANTHRACNOSE***

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FBSB 2014 19



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By

JEFFREY LIM SENG HENG

**Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfillment of the Requirements for the Degree of Doctor of Philosophy**

November 2013

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Abstract of thesis presented to the Senate of University Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Chairman: Associate Professor Umi Kalsom Md. Shah, PhD
Faculty: Biotechnology and Biomolecular Sciences

Red chilli is one of the most important diets for Malaysian. However chili plants are susceptible to attacks by few diseases such as anthracnose and Chili Mozaic Virus (CMV). Anthracnose is being considered as the major disease due to the ability of the fungi to attack both pre and post harvest chili fruits. With the high impact of anthracnose on chilli fruits the use of chemical pesticides has been frequent and uncontrolled. The high usage of chemical pesticides has caused damage not only to the environment but also human health. Biological control has been suggested as an alternative to counter the effect of chemical pesticides. Actinomycetes especially those from the genus of streptomycetes have been well known as a potential biological control agent for most of the plant pathogens. This study was conducted with the aim to investigate the characteristics of *Streptomyces ambofacines* S2 and to isolate antifungal compounds produced towards anthracnose for use as a biopesticide.

Colletotrichum capsici and *Colletotrichum gleosporioides* were isolated from infected chilli fruits using surface sterilization method. The fungi were later identified by targeting its internal transcribed spacer region (ITS). Molecular method confirmed that both *C. capsici* and *C. gleosporioides* were isolated.

A total of 513 isolates of actinomycetes were isolated from 5 different soil samples from both Peninsular Malaysia and Sabah and Sarawak using soil dilution method. The antifungal activity was detected using disc diffusion method. Through disc diffusion testing, 6 potential isolates of *Streptomyces* spp. were chosen for further studies (morphology study, molecular identification and carbon sources utilization analysis). However, only *Streptomyces ambofaciens* S2 was chosen for cultural condition optimization and bioactive compound isolation due to the highest inhibition zone exhibited towards *C. capsici* (15 mm) and *C. gleosporioides* (16 mm).

Metabolomic study conducted showed that *Streptomyces* spp. with antifungal producing abilities tend to produce more valine, isoleucine, leucine, asparagines, succinic acid and fructose, while non antifungal producing *Streptomyces* spp. produces more fatty acid, lactic acid, β glucose and gallic acid. Differences in the metabolite constituents have enable the used of metabolomic to characterize antifungal producing *Streptomyces* spp. from non antifungal producing *Streptomyces* spp.

The impact of media composition and cultural conditions were conducted for *S. ambofaciens* S2 using shake flask fermentation. It was observed that *S. ambofaciens* S2 produced the highest antifungal compound when chitin and peptone were used as the carbon and nitrogen sources respectively, 0.2% NaCl, 3 days incubation time, 6 days seed age and initial pH 8 for the broth. An increased of 33% in the inhibition zone was noted when *S. ambofaciens* S2 was grown using this condition. Chitinase activity was observed to be the highest when the culture was incubated for 7 days with the activity of 0.192 U/ml. Specific activity at day 7 was recorded as 1.28 U/mg. Formulation of an oil based liquid biopesticide using 20% glycerol added to the Chitin Peptone Media showed that *S. ambofaciens* S2 extract was able to give an effectiveness of 80% when tested *in vivo* for controlling chilli anthracnose.

Isolation of the bioactive compounds, indicated that the active compounds were eluted when 1:1 (hexane : ethyl acetate) was used. This active fraction was then subjected to purification using high pressure liquid chromatography (HPLC). The compound was white in colour when it was dried. Under liquid chromatography mass spectroscopy (LCMS) analysis, the compound was observed with the mass of 777.11 m/z. The compound was categorized under the group of polyenes.

Results from this study demonstrated *Streptomyces ambofaciens* S2 could be used as an alternative pesticide for controlling anthracnose in chilli fruits. In addition, metabolomic method could be used as a new way of fast characterization of different species of streptomycetes.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PENCIRIAN STREPTOMYCES AMBOFACINES S2 DAN PENERANGAN
KOMPOUN ANTIKULAT UNTUK KAWALAN BIOLOGI TERHADAP
ANTRAKNOS CILI**

Oleh

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November 2013

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Cili merah merupakan satu diet makanan yang penting untuk warga Malaysia. Walaubagaimanapun, pokok cili adalah tidak rintang terhadap serangan penyakit seperti antraknos dan penyakit mozek virus (CMV). Serangan antraknos adalah dianggap sebagai salah satu penyakit yang serius kerana kulat itu dapat menyerang buah cili sebelum dan selepas tuai. Di sebabkan oleh impak tinggi antraknos terhadap buah cili, penggunaan pestisida telah menjadi kerap dan tidak terkawal. Penggunaan pestisida kimia yang tinggi telah menyebabkan kerosakkan pada alam sekitar dan juga kesihatan manusia. Kawalan biologi telah di sarankan sebagai satu langkah alternatif untuk memerangi kesan pestisida kimia. Bakteria yang dikenali sebagai aktinomiset terutamanya genus *Streptomiset* adalah dikenalpasti sebagai agen kawalan biologi semulajadi untuk kebanyakan penyakit tumbuhan. Kajian ini telah dijalankan bagi pencirian *Streptomiset ambofaciens* S2 dan penyaringan kompaun antikulat terhadap antraknos bagi penggunaan sebagai biopestisida.

Colletotrichum capsici dan *Colletotrichum gleosporioides* telah di pencirkan daripada buah cili yang dijangkiti penyakit antraknos menggunakan kaedah pensterilan permukaan. Kulat yang diperolehi kemudiannya dikenalpasti dengan mensasarkan kawasan "internal transcribed spacer". Kaedah molecular mengesahkan bahawa kedua-dua *C. capsici* dan *C. gleosporioides* telah dipencilkan.

Sejumlah 513 isolat aktinomiset telah diperolehi daripada 5 sampel tanah yang diambil daripada Semenanjung Malaysia dan juga Sabah dan Sarawak dengan menggunakan teknik pencairan tanah bersiri. Aktiviti antikulat dikesan melalui penggunaan teknik "disc diffusion". Melalui teknik ini, 6 isolat *Streptomiset* spp. berpotensi telah dipilih untuk kajian lebih mendalam (moforlogi, pengenalpastian secara molekular dan analisis penggunaan bahan karbon). Bagaimanapun hanya *Streptomyces ambofaciens* S2 yang dipilih untuk dikaji pengoptimuman kondisi kultur dan pemencilan kompaun bioaktif kerana penghasilan zon perencat yang paling besar terhadap *C. capsici* (15 mm) dan *C. gleosporioides* (16 mm).

Kajian metabolomik yang dijalankan terhadap *Streptomiset* spp. yang menghasilkan antikulat menunjukkan lebih banyak valina, iso leusina, leusina, asparagina, asid suksinik and fruktos dihasilkan manakala *Streptomiset* spp. yang tidak menghasilkan antikulat menunjukkan penghasilan asid lemak, asid laktik, β -glukos dan asid galik yang lebih banyak. Perbezaan metabolit ini telah membolehkan keupayaan streptomiset untuk menghasilkan aktiviti antikulat terhadap antraknos.

Impak komposisi media dan kondisi kultur telah dijalankan bagi *S. ambofaciens* S2 dengan menjalankan fermentasi menggunakan kelalang kon. Daripada kajian ini, didapati bahawa *S. ambofaciens* S2 menghasilkan aktiviti antikulat pada tahap tertinggi apabila kitin dan pepton digunakan sebagai bahan karbon dan nitrogen, 0.2% NaCl, tempoh pengeraman selama 3 hari, umur sel 6 hari dan pH awalan 8. Penggunaan kondisi ini membolehkan peningkatan zon perencat sebanyak 33%. Aktiviti kitin didapati berada pada tahap tertinggi pada hari ke-7 pengeraman dengan aktivitinya dicatatkan pada 0.192 U/ml. Aktiviti khusus enzim yang diperolehi pada hari ke-7 adalah 1.28 U/mg. Formulasi minyak biopestisid cecair yang dihasilkan daripada ekstrak *S. ambofaciens* S2 dengan campuran 20% gliserol kepada “Chitin Peptone Media” menunjukkan keberkesanan sebanyak 80.0% pada cili yang diuji terhadap antraknos.

Penyaringan bahan bioaktif menunjukkan kompoun aktif diperolehi dengan penggunaan 1:1 (hexane : etil acetat). Kompoun aktif ini seterusnya dibersihkan dengan menggunakan kromatografi cecair prestasi tinggi (HPLC). Penggunaan kromatografi cecair spektrometri jisim (LCMS), memberikan jisim kompoun pada 777.11 m/z. Kompoun bioaktif ini di kelaskan pada kelas “polyenes”.

Keputusan daripada kajian ini menunjukkan *Streptomyces ambofaciens* S2 boleh digunakan sebagai pestisid alternatif untuk antraknos buah cili. Tambahna daripada ini, kajian metabolomik boleh digunakan sebagai satu cara baru untuk pencirian spesies streptomiset yang berlainan.

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I certify that a Thesis Examination Committee has met on 27th November 2014 to conduct the final examination of Jeffrey Lim Seng Heng on his thesis entitled “**CHARACTERIZATION OF *STREPTOMYCES AMBOFACIENS* S2 AND ELUCIDATION OF ITS ANTIFUNGAL COMPOUNDS FOR BIOCONTROL OF CHILLI ANTHRACNOSE**” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
μl	microlitter
C	Carbon
Ca	Calcium
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	Calcium Nitrate-4 hydrate
CaCO_3	Calcium Carbonate
cfu	Colony Forming Unit
cm	Centimeter
D	Dextro
dH ₂ O	Distilled water
DNA	Deoxyribonucleic Acid
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	Ferrous Sulphate-7 hydrate
FTIR	Fourier Transformed Infrared
g	Gram
G + C	Guanine + Cytosine
H	Hydrogen
h	Hour
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
KCl	Kalium Chloride
KH_2PO_4	Kalium dihydrogen phosphate
KNO_3	Kalium Nitrate
L	Levo
L	Litter
LCMS	Liquid Chromatography Mass Spectroscopy
M	Molarity
mg	Milligram
ml	Mililiter
mm	milimeter
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	Magnesium Sulphate-7 hydrate
min	Minute
m/z	Mass to charge ratio
Na_2HPO_4	<i>di</i> -natrium hydrogen phosphate
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	<i>di</i> -natrium hydrogen phosphate-12 hydrate
NaCl	Natrium Chloride
$(\text{NH}_4)_2$	<i>di</i> -ammonium
NMR	Nuclear Magnetic Resonance
OPLS	Orthogonal Partial Least Square
PCA	Principle Component Analysis
PCR	Polymerase Chain Reaction
pH	Potential of hydrogen

ppm	Part per million
sec	Second
SEM	Scanning Electron Microscope
spp.	Species
w/v	Weight per volume
v/v	Volume per volume
%	Percentage
°C	Degree Celsius



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CHAPTER 1

INTRODUCTION

Red chilli or scientifically known as *Capsicum annum* has been a part of Malaysian daily meal diet. The use of red chilli fruits include for the making of sambal (a kind of spicy chilli sauce make from dried red chilli and onion) and curry rendang (a Malay delicacy prepared from curry and chicken or beef). According to FAO report, 2012, area cultivated with chilli in Malaysia in the year 2000 was estimated to be around 2,200 ha with estimated yield of 113,636.36 Hg/Ha (FAOSTAT, 2012). This number increased to 2,594 ha in the year 2010 with the total estimated yield of 134,244.41 Hg/Ha (FAOSTAT, 2012). This has showed an increased of approximately 15 % of the total cultivated area and hence indicating the importance of the crop.

Like any other plants, chilli has two most prevalence diseases namely the chilli mosaic virus (CMV) and anthracnose (Than *et. al.*, 2008a; Isaac, 1992). Both diseases have been reported to cause yield loss and reduce in the marketability of the chilli fruits (Iqbal *et al.*, 2012; Suwan *et al.*, 2012). Anthracnose is considered to be more severe due to the fact that anthracnose could infect the chilli fruits both pre and post harvest. According to Than *et al.* (2008a), anthracnose disease can infect the whole chilli plant itself. Typical symptoms of anthracnose were leaf tip die-back, stem die-back, foliar blight, leaf spot, leaf lesion and for fruit there would be sunken necrotic tissues, with concentric rings of acervuli that are often wet. Chemical methods used to prevent the occurrence of anthracnose disease might cause health hazard to the consumers. Due to that biological control methods would be considered the best practice (Pal and Gardener, 2006).

Actinomycetes are Gram-positive bacteria which have been long known for its special morphological criteria (Abou-Elela and Ghanem, 2005). Actinomycetes are the only bacteria with the capacity to form branches of ramifying network of filaments which is also known as mycelium. Apart from the mycelium, actinomycetes also produce abundant asexual spores known as conidia. These conidia gave the actinomycetes colonies their ‘powdery’ look on the agar media plate. Due to this special characteristic actinomycetes are also called pseudo-bacteria. The name actinomycetes come from two Latin words, actinis which means ‘ray’ and myces which means fungus (Hopwood, 2007).

Actinomycetes are classified as bacteria due to several factors such as the present of peptidoglycan in their cell wall, their sensitivity towards lysozyme that will degrade the polysaccharide backbone of the peptidoglycan and their sensitivity towards antibacterial but not antifungal antibiotics. Actinomycetes can be differentiated into different genera based on their morphological, physical and chemical criteria (George *et al.*, 2010).

Malaysia which had been known as one of the world mega diversity country has the potential of obtaining novel microorganisms with new bioactive compounds (Jeffrey, 2008). Actinomycetes had been known to be most abundant in soil compared to water and air (Kumar *et al.*, 2010; Jeffrey, 2008; Thangapandian *et al.*, 2007). The ability of microorganisms to produce bioactive compounds have been related to their environmental stresses. These stresses caused the microorganisms to produce enzymes that could help them to combat the stresses. There are 2 kinds of enzymes system produced by the microorganism; intracellular and extracellular. Extracellular enzymes or exoenzymes are more easily isolated as no rupture of bacterial cells were needed. This has helped the manufacturers to reduce their production cost due to easy recovery of enzymes from the bacterial cells (Asad *et al.*, 2011).

Isolation of bioactive compounds from actinomycetes had been well documented by researchers worldwide (Kumar *et al.*, 2010; Ramesh and Mathivanan, 2009; Jeffrey, 2008; Thangapandian *et al.*, 2007). The most distinguished genera of actinomycetes that had been widely isolated for their secondary metabolites activities are Streptomycetes. *Streptomyces* spp. are easily found in soil and they contributed to the soil microbial communities in many way such as degradation of polysaccharides present in the soil for easily uptake of nutrients by plants and other microorganisms and secretion of bioactive compounds to inhibit the growth of certain microorganisms. The first isolated antibiotic from actinomycetes was streptomycin an antibiotic isolated from *Streptomyces griseus* (Waksman *et al.*, 1946). Streptomycetes had been well known to secrete majority antifungal compounds from the group of polyene and macrolides.

The ability of microorganisms to produce their metabolites would depend on their ability to modulate their metabolic composition according to their environment. By understanding the metabolites produced, researchers are able to study the unique cellular process of certain microorganisms which may be influence by physiological and also environmental signals (Jensen *et al.*, 2006). Fingerprinting these chemical constituents that present in the microorganism may helps to further understand the microorganism antibiotic pathway.

With the increasing demand for chilli and the effect of chemical pesticides towards human health, this research was done to identify a biocontrol agent that could be used as an alternative for the current practices for chilli planters. In the current practice, chemical pesticides have been used heavily to control pest and disease in chilli, this has caused contamination to the environment (Sharma and Parihar, 2010). The use of chemical pesticides do not just caused contamination but also creating a more resistance and mutated microorganisms that needs higher dosage of the chemical substances. The use of natural occurring enemies or biological control could help to minimize the impact of the chemical pesticides in agriculture (Pal and Gardener, 2006). Utilization of antagonistic microorganisms has been found in postharvest of various fruits and vegetables (Fravel, 2005). The used of biological control agents in post harvest have been reported to be very efficacious for controlling anthracnose on chilli (Chanchaichaovivat *et al.*, 2007). The

main objective of this study was to reduce the used of the chemical pesticides by obtaining a biological based pesticide for controlling anthracnose in chilli.

In this study, we isolated streptomycetes from the soil samples collected and then proceed with study on the impact of cultural condition on the potential streptomycetes. The antifungal compound obtained from the potential actinomycetes were then isolated and characterized. Metabolomic study was performed to determine the metabolites responsible for antifungal production (Appendix 1.1). It is hypothesized that secondary metabolite produce by selected streptomycetes were an extracellular compound and this compound inhibited the growth of *Colletotrichum gloeosporioides* through the mode of antibiosis.

The specific objectives of this study were:-

- 1) To isolate, screen and characterize streptomycetes with the ability to produce antifungal activity.
- 2) To characterize metabolites presents for both antifungal and non antifungal producing *Streptomyces* spp. using metabolomic study.
- 3) To investigate the impact of medium composition (carbon, nitrogen, NaCl) and cultivation conditions (pH, seed age, agitation, temperature) on the production of antifungal activity by *Streptomyces ambofaciens* S2.
- 4) To characterize the antifungal compounds produced by *Streptomyces ambofaciens* S2.

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