UNIVERSITI PUTRA MALAYSIA

LIGNO-CELLULOSE DEGRADING AND ANTIMICROBIAL ACTIVITIES OF SELECTED STRAINS OF ACTINOMYCETES ISOLATED FROM MALAYSIAN RAINFOREST SOILS

TANG SUI YAN

FSTM 2006 24

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By

TANG SUI YAN

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

December 2006

DEDICATION

I dedicate this work to my dear late grandmother and beloved friend, Lucky.

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

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DECEMBER 2006

Chairman: **Professor Son Radu, PhD**

Faculty: **Food Science and Technology**

Eighteen soil samples were collected from the tropical forest floor at Kuala Tahan National Park, Pahang. Soil Water content and soil pH were determined. Isolation of actinomycetes bacteria isolates was accomplished *via* multi-stage Dispersion and Differential Centrifugation (DDC) technique and total of 227 isolates were obtained and maintained on Starch-Casein Nitrate (SCN) medium. All soil samples gave high colony forming units (C.F.U.) counts when grown on medium of pH 7.0 (1.0-6.7 x 10^6) as compared to pH 5.0 (0.4-2.2 x 10^6). All isolates showed very diverse colony morphologies. Of 227 isolates, 24.67% (56/227) showed Xylanase activity, 28.63% (65/227) with Cellulase activity and 7.93% (18/227) for Galacto-mannanase. Antagonistic activities were also observed against plant pathogens of *Xanthomonas campestris* at 12.78% (29/227), *Ralstonia solanacearum* at 39.65% (90/227), *Erwinia crysanthemi* 0% (0/227) and *Pantoea stewartii* at 16.30% (37/227). Whereas in gram

negative food pathogens of *Salmonella typhimurium* at 0.88% (2/227) and *Vibrio parahaemolyticus* at 6.61% (15/227). Antagonistic against gram positive food pathogens of *Staphylococcus aureus* was at 21.59% (49/227) and *Listeria monocytogenes* at 6.61% (15/227). Ten actinomycetes isolates (A3, C1, G2, G3, G4, G10, I15, L8, O15 and P5) were selected based on their bioactive compound profiles and subjected to microscopic studies, metabolic finger printing using BIOLOG system and partial 16S rDNA analysis. Significant and diverse differences between all 10 selected isolates were observed *via* BIOLOG carbon utilization profiling and cell morphology under light microscopy. Isolate G4 and O15 were identified as members of genus *Kitasatospora* while the other eight isolates belong to genus *Streptomyces* using 16S rDNA sequence analysis and light microscopy. The ability to produce extra-cellular enzymes and antagonism activity against plant pathogens indicated that the 10 selected isolates have potential to be consortia of microorganisms as inoculum in agro-waste composting such as oil palm's empty fruit bunch. The inoculum not only increases the nutrient value in compost materials but also has advantage to control plant disease in soil ecosystem.

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

AKTIVITI PENGURAIAN "LIGNO-CELLULOSE" DAN ANTIMIKROBIAL STRAIN TERPILIH ACTINOMYCETES DIPENCIL DARIPADA TANAH HUTAN HUJAN TROPIKAL MALAYSIA

Oleh

TANG SUI YAN

DISEMBER 2006

Pengerusi: **Professor Dr. Son Radu, PhD**

Fakulti: **Sains dan Teknologi Makanan**

Lapan belas sampel tanah telah dikumpulkan dari lantai hutan tropika di Taman Negara Kuala Tahan, Pahang. Kadungan air tanah dan pH tanah telah ditentukan. Pemencilan bakteria actinomycetes isolat dicapai melalui teknik "Dispersion and Differential Centrifugation" (DDC) yang berperingkat dan kesemuanya 227 kultur isolat telah dicapai dan diperlihara atas "Starch-Casein Nitrate" (SCN) media. Semua sampel tanah memberikan kiraan C.F.U. yang tinggi semasa ditumbuhkan atas media dengan pH 7.0 (1.0-6.7 x 10^6) berbanding dengan pH 5.0 (0.4-2.2 x 10^6). Kesemua isolat telah menunjukkan morfologi koloni yang amat pelbagai. Daripada 227 isolat, 24.67% (56/227) menunjukkan aktiviti "Xylanase", 28.63% (65/227) dengan aktiviti "Cellulase" dan 7.93% (18/227) untuk "Galacto-mannanase". Aktiviti antagonistik juga telah diperhatikan terhadap patogen tumbuhan *Xanthomonas campestris* pada 12.78% (29/227), *Ralstonia solanacearum* pada 39.65% (90/227), *Erwinia*

crysanthemi 0% dan *Pantoea stewartii* pada 16.30% (37/227). Manakala dalam patogen makanan gram negatif *Salmonella typhimurium* pada 0.88% (2/227) dan *Vibrio parahaemolyticus* pada 6.61% (15/227). Antagonistik terhadap patogen makanan gram positif *Staphylococcus aureus* adalah pada 21.59% (49/227) dan *Listeria monocytogenes* pada 6.61% (15/227). Sepuluh actinomecetes isolat (A3, C1, G2, G3, G4, G10, I15, L8, O15 dan P5) telah dipilih berdasarkan profil bioaktif kompaun mereka dan ditujukan kepada pemerhatian mikroskopik, "metabolic finger printing" menggunakan sistem BIOLOG dan analisis separa 16S rDNA. Perbezaan yang ketara dan pelbagai antara kesemua 10 isolat terpilih telah diperhatikan melalui profil penggunaan karbon BIOLOG dan morfologi sel di bawah mikroskop cahaya. Isolat G4 dan O15 telah dikenalpasti sebagai ahli genus *Kitasatospora* manakala lapan isolat yang lain kepunyaan genus *Streptomyces* menggunakan analisis 16S rDNA and pemerhatian mikroskopik cahaya. Kebolehan untuk menghasilkan enzim di luar cell dan aktiviti antagonistik terhadap patogen tumbuhan menunjukkan bahawa 10 isolat terpilih mempunyai potensi dijadikan konsortia mikroorganisma sebagai inoculum dalam kompos sisa pertanian seperti "empty fruit bunch" kelapa sawit. Inoculum ini bukan sahaja menambakan nilai nutrasi dalam bahan kompos tetapi juga mempunyai kelebihan untuk mengawal penyakit tumbuhan dalam ekosistem tanah.

ACKNOWLEDGEMENTS

Through out my master study, I received much supports and guidance from various parties that had helped me greatly in completing my work.

First and for most, I am truly indebt to my supervisory committee members, Chairman Professor Dr. Son Radu, Co-supervisor Dr. Sahilah Abd. Mutalib and Associate Professor Suhaimi Napis. I am deeply grateful for I would not be able to accomplish this thesis without their continual guidance, supports and constructive advice throughout my master study.

I like to express my indebtedness to the Malaysian Agriculture Research and Development Institute (MARDI) for allowing me to conduct my research at their facilities. Thanks really must go to MARDI's research officers, Pn. Tosiah Sadi and Mr. Jeffrey Lim Seng Heng for their encouragements, sound judgements and valuable suggestions. MARDI's staffs Pn. Rosnah Hassan, Puan Norzaimawati Binti Aman Nejis and Mr. Noor Azli Hashim who offered their assistant during the soil sample collection expedition at National Park, Kuala Tahan, Pahang. I cherish all your help, patient and sumptuous hospitality given to me when I was conducting my lab work in Microbiology Laboratory, that enable me to accomplished my task with no complication.

Most importantly is my deepest gratitude to the Institute of Strategic and International

Studies (ISIS) Malaysia for offering me the Perdana scholarship, under the Malaysia Prime Minister Fellowship Exchange Programme session 2004/2005. The sponsorship had enabled me to complete my study free from any financial difficulties. I wish to thanks the director of Perdana Scholarship, Mr. Mohamed Md. Ibrahim and the officers Ms. Azza, Mr. Rostam and Mr. Rosli, for their guidance and full assistance.

Also I wish to show my gratitude to the Department of Wildlife and National Parks, Peninsular Malaysia (PERHILITAN) for their full cooperation and permission to conduct the soil sample collection expedition during 28th till 30th April 2004.

Not forgetting my dearest friends, Afifah Abd. Rahman and Jacintha Sugnaseelan, although we all apart but I always cherish and treasure the ups and downs, sweet sour that we all experience in our persuade of postgraduate studies. I also like to express my appreciation to my course mates in Faculty of Food Science and Technology for their friendship and supports.

Finally to my family and Yoon Wai for they had bare with my frequent absent and ignorant all this while.

I certify that an Examination Committee has met on 15th December 2006 to conduct the final examination of Tang Sui Yan on her Master of Science thesis entitled "Ligno-Cellulose Degrading and Antimicrobial Activities of Selected Strains of Actinomycetes Isolated from Malaysian Rainforest Soil" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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

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Date:12th APRIL 2007

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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TANG SUI YAN

Date: 12th APRIL 2007

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LIST OF ABBREVIATIONS

~	Approximately
β	Beta
<	Less than
≤	Same or less than
≥	Same or more than
%	Percentage
µg	Microgram
µl	microlitre
rDNA	Ribosomal Deoxyribonucleic Acid
C	Cytosine
C.F.U.	Colony Forming Unit
CO_2	Carbon dioxide
DDC	Dispersion and Differential Centrifugation
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside triphosphate
Dry wt	Dry soil weight
EtBr	Ethidium Bromide
G	Guanine
Gram +ve	Gram positive
H^+	Hydrogen ion
H_2	Hydrogen gas
HCl	Hydrochloric acid

HCO_3^-	Acid Carbonate
KAc	Potassium Acetate
KCl	Potassium Cloride
M	Molar or molarity (moles of solute per litre of solution)
MARDI	Malaysian Agriculture Research and Development Institute
mg	Milligram
$MgCl_2$	Magnesium Chloride
min	minute(s)
mM	milli Molar
mol	mole
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
ºC	Degree centigrade
PCI	Phenol-Chloroform-Isoamyl alcohol
PCR	Polymerase Chain Reaction
PD wt	Petri dish weight
pH	Concentration of Hydrogen ion
R	Residue
RNA	Ribonucleic acid
rDNA	Ribosomal Deoxyribonucleic acid
rpm	revolution per minute
S	Svedberg unit
S(n)	Supernatant fraction no.

SCN	Starch-Casein Nitrate Media
SDS	sodium dodecyl sulphate
spp.	Species
SWC	Soil water content
Taq	*Thermus aquaticus* DNA polymerase
TBE	Tri-Borate EDTA electrophoresis buffer
Tris	Tris (hydroxymethyl) methylamine
UV	ultraviolet
v/v	volume/ volume
Wet wt	Wet soil weight
w/v	weight/ volume

CHAPTER 1

INTRODUCTION

Actinomycetes bacteria are widely distributed in nature and they thrive in soil where they play an important role in the bioremediation, mineralization and decomposition of organic matter with the production of numerous extra-cellular enzymes such as amylases, chitinases, cellulases and proteases. The potential and application of actinomycetes in biodegradation and composting process of agriculture and urban wastes were investigated and being successful (Fergus, 1964; Crawford, 1988; Lacey, 1997; Chamberlian and Crawford, 2000). Actinomycetes incorporated into composting of palm oil empty fruit bunch (Thambirajah *et al.*, 1995) and green waste compost (Lacey, 1997) were proven their potential with viability remain quite high throughout composting for both mesophilic and thermophilic strains beside improved degradation process.

At the same time, extensive studies had also been conducted to evaluate the possibility of actinomycetes species as biological control of numerous plant pathogens in various vegetations and in compost (Johnson, 1954; Mehrotra and Caludius, 1972; Hardy and Sivasithamparam, 1995; Yuan and Crawford, 1995; Cao *et al.*, 2004). Studies in

evaluating the growth inhibition of animal and plant pathogens by actinomycetes bacteria isolated from soil samples of different regions of the world were also increased significantly in the past decade (Erwealor and Njoku-Obi, 1990; Ndonde and Semu, 2001; Barakate *et al*., 2002; Lo *et al*., 2002; Moncheva *et al*., 2004; Oskay *et al.,* 2004).

The idea of introducing antagonistic actinomycetes in compost may offer an interesting alternative. Inoculation of compost with actinomycetes provides many promising benefits and added advantages in enabling the production of suitable, stable and desirable compost composition by enzyme degradation; as well as biological control agent against human and plant pathogens present in the natural field environment (Hardy and Sivasithamparam, 1995). This may helps open up the badly needed agricultures market for farmers, compost producers and the environment where mass production of stable and inexpensive development of a microbial biological control agent will be possible (Cook, 1993), plus tonnes of agricultural waste can also be transformed into environmentally friendly biofertilizers.

It is well understood that soil and particularly forest soil is an excellent and massive source of actinomycetes bacteria diversity (Xu *et al*., 1996). Based on previous study by Lo *et al*. (2002) and Numata and Nimura (2003), actinomycetes bacteria isolated from Malaysian forest soils were very high in morphological variety and suggesting

the vast diversity of local actinomycetes bacteria strains. Currently there were little documentation of Malaysian actinomycetes and their potential to produce secondary metabolites (Vikineswary *et al*., 1997; Ismet *et al*., 2002; Lo *et al*., 2002; Numata and Nimura, 2003). Isolates obtained from such unexplored environment may be looked upon as being wild types showing natural variability (Ndonde and Semu, 2001; Barakate *et al*., 2002).

In this master thesis, I had decided to investigate the unexplored tropical rainforest soil from Malaysian National Park, Pahang as the source of actinomycetes bacteria. Local isolates with desired lignocellulytic enzyme activities and antimicrobial activities against numerous plant and human pathogens will be selected through preliminary screening process. And later selected actinomycetes as potential inoculum for composting and biofertilizer development shall be further characterize and identified to genus level.

The main objectives and aim of my study are summarized as:

1. To collect soil samples from Malaysian low land tropical forest (National Park, Pahang);
2. To isolate actinomycetes bacteria using modified isolation method;
3. To screen for biological activities of secondary metabolites (enzymes and anti-microbial activity);

4. To select actinomycetes isolates with useful bioactive compound profiles;
5. To identify and characterise the potential actinomycetes isolates *via* Biolog Identification System for metabolic fingerprinting, microscopy studies under light microscope and PCR-amplified 16S rDNA profiling.

This research work will serves as a basic and fundamental data on the isolation and characterization of Malaysian soil actinomycetes for the potential development of inoculum for agricultural waste composting in future.

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy of actinomycetes genus

Actinomycetes are Gram +ve, heterotrophic prokaryote, belong to the order *Actinomycetales* (Lechevalier and Lechevalier, 1980). Member of genus Actinomyces are anaerobic and facultative anaerobic (Hall *et al*., 1999).These bacteria are 0.5 - 1.0 µm in size and phylogenetically, defined as a number of taxa within the high G+C (60-70 mol %) subdivision of gram-positive phylum. The name Actinomycetes derived from Greek word Aktino meaning ray, and mykes meaning mushroom/ fungus, owing to formation of its filamentous and sporulating colonies. Thus they are recognised as a transition group between primitive bacteria and fungi.

Actinomycetes are slow growing bacterial. They require incubation for 1 – 3 weeks or more at 25 – 35 °C and 45 – 55 °C for mesophilic strains and thermophilic strains respectively. Cell cycle starts with germination of spore and grows by forming branching filaments of cells which become a network of strands called a vegetative