CHARACTERIZATION OF *Trichoderma* ISOLATED AND THEIR LIGNOCELLULOLYTIC ACTIVITIES

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JUDUL: **CHARACTERIZATION OF *Trichoderma* ISOLATED AND THEIR LIGNOCELLULOLYITC ACTIVITIES**

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DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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ABSTRACT

Trichoderma is a cosmopolitan fungus that prevalent in the soil and other diverse habitats. It has gained vast economical importance's because industrial enzymes production, antifungal, antibiotics, biocontrol agents and plant growth promoter. *Trichoderma* species produced the lignocellulolytic enzymes activities that assisted in the degradation of woody lignocellulose materials for industrial applications. Therefore, an accurately identification of *Trichoderma* isolates at the species level is highly desirable. In this study, the main aim to accurately identify of *Trichoderma* isolates at the species level based on the morphological characteristics, lignocellulolytic enzyme activities and multilocus gene sequencing based on the Internal Transcribed Spacers 1 and 2 (ITS1 and ITS2) regions of the rDNA, a partial sequence of the Translation Elongation Factor 1-alpha (*tef1*) and calmodulin (*cal*). A total of 53 isolates of *Trichoderma* were isolated from wet paddy field in Tuaran, Sabah, Malaysia. On the combination of morphological characteristics and multilocus gene sequencing analysis were positively identified three *Trichoderma* species, namely *T. asperellum*, *T. harzianum* and *T. reesei*. The phylogenetic relationships were constructed based on the Internal Transcribed Spacers 1 and 2 (ITS1 and ITS2) regions of the rDNA gene, a partial sequence of the Translation Elongation Factor 1-alpha (*tef1*) and calmodulin gene (*cal*) by using UPGMA method and found three sections such as *T. asperellum* in the "*Trichoderma*" section, *T. harzianum* in the "*Pachybasium*" section, and *T. reesei* in the "*Longibrachiatum*" section. Mycelial growth and biomass yield among three species isolates were examined on Potato Dextrose Agar and Potato Dextrose Broth, respectively, with different temperatures; 20, 25, 30, 35 and 40 °C. *T. asperellum* isolates were grown very well of mycelia growth and produced the highest biomass yield at 30 °C, followed by *T. harzianum* and *T. reesei*. The lignocellulolytic activities were assayed based on their ability to develop dark brown pigments, yellow halo zone, and clear white zone on tannic acid media (TAM) for lignin, Jensen Media (JM) for cellulose and modified Melin–Nokrans media (MMNM) for starch. The diameters of halo zones were measured for the analysis of their ability in degrading lignin, cellulose, and starch. The best seven *Trichoderma* isolates [S1(9)10^{-1}(3), E3(6)10^{-1}(2), W2(2)10^{-1}(2), S3(1)10^{-1}(1), N2(4)10^{-2}(3), N2(2)10^{-1}(2) dan S3(6)10^{-1}(2)] were found the potential lignocellulolytic agents based on the diameter of dark brown pigments and halo zones formed. *Trichoderma* species are found to synthesize polyphenol oxidase, endoglucanases, and are able to hydrolyze starch to glucose in the three different media. Thus, the potential *Trichoderma* isolates can be further applied as biocontrol agents in controlling disease and increasing yield of agricultural crops.

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ABSTRAK

MORFOLOGI, LIGNOSELLULOLISIS DAN PENCIRIAN MOLEKUL PENCILAN TRICHODERMA DARIPADA SAWAH PADI, TUARAN

Trichoderma adalah kulat kosmopolitan yang tersebar luas di dalam tanah dan pelbagai habitat lain. Ia mempunyai kepentingan dalam ekonomi kerana pengeluaran enzim untuk industri, anti-kulat, antibiotik, agen kawalan biologi dan membantu pertumbuhan tumbuhan. Spesies Trichoderma menghasilkan enzim lignosellulolisis yang membantu dalam degradasi lignoselulosa bahan berkayu untuk diaplikasikan dalam industri. Oleh itu, pengenalan pastian Trichoderma yang tepat di peringkat spesies adalah sangat diperlukan. Dalam kajian ini, tujuan utama adalah untuk mengenal pasti dan mengasingkan Trichoderma dengan tepat di peringkat spesies berdasarkan ciri morfologi, aktiviti enzim lignocellulolisis dan multilokus jujukan gen berdasarkan Internal Transcribed Spacers 1 and 2 (ITS1 and ITS2) kelompok rDNA, sebahagian daripada jujukan Translation Elongation Factor 1-alpha (tef1) and calmodulin (cal). Sebanyak 53 pencilan Trichoderma telah diasingkan daripada sawah padi di Tuaran, Sabah, Malaysia. Berdasarkan gabungan ciri morfologi dan analisis multilokus jujukan gen, ia secara positif telah mengenal pasti tiga spesies Trichoderma iaitu T. asperellum, T. harzianum dan T. reesei. Hubungan filogenetik telah dibina berdasarkan disalin Internal Transcribed Spacers 1 and 2 (ITS1 and ITS2) kelompok rDNA, sebahagian daripada jujukan Translation Elongation Factor 1-alpha (tef1) and calmodulin (cal) gen dengan menggunakan kaedah UPGMA dan menunjukkan tiga bahagian iaitu T. asperellum dalam seksyen "Trichoderma", T. harzianum dalam seksyen "Pachybasium", dan T. reesei dalam seksyen "Longibrachiatum". Pertumbuhan miselium dan hasil biojisim antara tiga spesies telah diuji ke atas Potato Dextrose Agar dan Potato Dextrose Broth, masing-masing, dengan suhu yang berbeza; 20, 25, 30, 35 dan 40 °C. T. asperellum dapat tumbuh dengan dan menghasilkan pertumbuhan mycelia dan hasil biojisim yang paling tinggi pada 30 °C, diikuti oleh T. harzianum dan T. reesei. Aktiviti lignocellulolisis telah dicerakinkan berdasarkan keupayaan mereka untuk menghasilkan pigmen coklat gelap, halo zon kuning, dan zon putih yang jelas ke atas media asid tannic (TAM) untuk lignin, Jensen Media (JM) untuk selulosa dan Melin-Nokrans media (MMNM) untuk kanji. Diameter halo zon diukur untuk analisis keupayaan mereka dalam menguraikan lignin, selulosa, dan kanji. Tujuh pencilan Trichoderma [S1(9)10^{-1}(3), E3(6)10^{-1}(2), W2(2)10^{-1}(2), S3(1)10^{-1}(1), N2(4)10^{-2}(3), N2(2)10^{-1}(2) dan S3(6)10^{-1}(2)] telah dikenalpasti mempunyai potensi sebagai ejen lignocellulolisis berdasarkan diameter coklat gelap dan zon halo yang terbentuk. Spesies Trichoderma mampu mensintesis poliphenol oxidase, endoglucanases, dan dapat menghidrolisis kanji kepada glukosa dalam tiga media yang berbeza. Oleh itu, pencilan Trichoderma yang berpotensi boleh diaplikasikan sebagai agen kawalan biologi dalam mengawal penyakit dan meningkatkan hasil tanaman pertanian.

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LIST OF SYMBOLS AND UNITS

cfu.g^{-1}	-	Colony forming unit per gram
μM	-	Micrometer
mm	-	Milimeter
mg.mL^{-1}	-	Milligram per millilitre
ng	-	Nanogram
μg	-	Microgram
g	-	Gram
mL	-	Mililiter
μL	-	Microliter
mM	-	Milimolar
nm	-	Nanometer
min	-	Minute
sec	-	Second
°C	-	Degree celcius
%	-	Percentage
±	-	Plus minus
rpm	-	Rotation per minute
V	-	Voltan

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LIST OF ABBREVIATIONS

6PP	-	6-penthyl-alpha-pyr-one
BCA	-	Biological control agent
BLAST	-	Basic Local Alignment Search Tool
bp	-	Basepair
C/N	-	Carbon to nitrogen ratio
CAZy	-	Carbohydrate-active enzymes
CFU	-	Colony Forming Unit
CMC	-	Carboxymethyl cellulose
CTAB	-	Cetyltrimethylammonium Bromide
DNA	-	Deoxyribonucleotide acid
dNTP	-	Deoxynucleotides triphosphate
EDTA	-	Ethylenediaminetetraacetic acid
EFB	-	Empty fruit bunch
EtBr	-	Ethidium bromide
tef1	-	Translational elongation factor 1-α
ISTH	-	International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy
ITS 1	-	Internal transcribed spacer 1
ITS 2	-	Internal transcribed spacer 2
ITS 4	-	Internal transcribed spacer 4
JM	-	Jensen media
Kb	-	Kilo base

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LiPs	-	Lignin peroxidases
MEGA	-	Molecular Evolutionary Genetic Analysis
MMNM	-	Modified-Melin Nokrans media
MnPs	-	Manganese-peroxidases
NCBI	-	National Center for Biotechnology Information
PAHs	-	Polycyclic aromatic hydrocarbons
PCR	-	Polymerase Chain Reaction
PDA	-	Potato Dextose Agar
PDB	-	Potato Dextrose Broth
PPO	-	Polyphenol oxidase
rDNA	-	Ribosomal DNA
rRNA	-	Ribosomal RNA
Sect.	-	Section
SMs	-	Secondary metabolites
spp.	-	Species
TAM	-	Tannic acid media
TBE	-	Tris-Borate-EDTA
tRNA	-	Transfer ribonucleic acid
TSM	-	*Trichoderma* Selective Medium
UPGMA	-	Unweighted Pair Group with Arithmetic Mean

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

The genus *Trichoderma* belongs to ascomycetic (Ascomycota, Hypocreales) fungi found in various ecosystems such as agricultural fields, forest, salt marshes and deserts, in almost all climatic zones (Roiger, Jeffers and Caldwell, 1991; Samuels, 2006; Kumar, Amaresan, Bhagat, Madhuri and Srivasta, 2010). Some *Trichoderma* spp. have economic importance because of its potential producers of enzymes, antibiotics and used as a biocontrol agent in the agricultural field (Harman and Björkman, 1998; Monte, 2001). Thus, precise identification and characterization of these fungi is a vital requirement. The early approach for identification of *Trichoderma* is on the morphological basis (Rifai 1969; Bissett, 1984; Dodd, Lieckfeldt and Samuels, 2000). Morphological descriptions such as colony appearance and microscopic characteristics which include phialide and conidia sizes, conidiaphores and formation of chlamydospores were observed. However, morphological alone is insufficient to identify *Trichoderma* accurately, since they are genetically diverse and characterized by variable morphology particularly between the closely related species of *Trichoderma* (Chaverri and Samuels, 2003; Chaverri, Castlebury, Samuels and Geiser, 2003; Druzhinina, Kubicek, Komoń-Żelazowska, Mulaw and Bissett, 2010).

The advent in molecular methods and identification tools based on molecular data from DNA sequencing has led to satisfactory taxonomy identification. DNA-based methods based on DNA barcoding are now routinely used in *Trichoderma* identification. It has done based on multilocus DNA sequence analysis of internal transcribed spacers (ITS) 1 and 2 of the ribosomal deoxynucleic acid (rDNA), gene cluster and fragments of the translational elongation factor 1-α

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(*tef1*), RNA polymerase II subunit (*rpb2*), chitinase 18-5 (*chi18-5*), actin (*act*) or calmodulin (*cal*) (Kindermann, El-Ayouti, Samuels and Kubicek, 1998; Dodd *et* al., 2000; Druzhinina, Kopchinskiy, Komoń, Bissett, Szakacs and Kubicek, 2005; Gal-Hemed, Atanasova, Komoń-Żelaswoska, Druzhinina, Viterbo and Yarden, 2011; Blaszczyk, Popiel, Chelkowski, Koczyk, Samuels, Sobieralski and Siwulski, 2011; Atanasova, Druzhinina and Jaklitsch, 2013; Jaklitsch and Voglmayr, 2015). Most studies have been used the combination of ITS and *tef1* for the identification of new species of *Trichoderma* (Bissett, Szakacs, Nolan, Druzhinina, Gradinger and Kubicek, 2003; Kraus, Druzhinina, Gams, Bissett, Zafari, Szakacs, Kopchinskiy, Prillinger, Zare and Kubicek, 2004; Lu, Druzhinina, Fallah, Chaverri, Gradinger, Kubicek and Samuels, 2004). In addition, sequence data may useful for phylogeny study, providing valuable insights into their evolutionary relationships. Moreover, the correct identification serves as an efficient selection and use of such isolates for commercial applications.

Druzhinina *et al.* (2005) introduced an online program using oligonucleotide barcodes, based on ITS and *tef1* sequences, known as International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (ISTH). At present, the database has listed more than 100 species which have been identified based at the molecular level. The basis of the method is an oligonucleotide barcode generated from a diagnostic combination of various oligonucleotides of ITS1 and ITS2 sequences. These online tools have been allowed the identification of most *Trichoderma* isolates or suggest them as representatives of potentially new species.

Lignocellulolytic enzymes are produced by a wide variety of fungi, including species in *Trichoderma* genus. Some *Trichoderma* spp. are great hydrolytic enzyme producer and therefore important for the biotechnological industry, such as *T. reesei* and *T. viride* (Mandels, Weber and Parizek, 1971; Domigues, Queiroz, Cabral and Fonseca, 2000). The search for potential biomass degrading enzymes also led to the isolation of these fungi (Sallenave-Namont, Pouchus, du Pont, Lossus and Verbis, 2000). Lignocellulolytic fungi have a potential to degrade a range of the lignocellulosic biomass. Many lignocellulosic materials such as wood, bagasse and

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wheat straw have been studied as potential substrates for the production of lignocellulolytic enzymes (Duff and Murray, 1996; Ogel, Yarangumeli, Dundar and Ifrij, 2001; Kalogerist, Christakopoulos, Katapodis, Alexiou, Vlachou, Kekos and Macris, 2003). The purified lignocellulolytic enzymes are used for commercial applications such as in coffee production where the hydrolysis of cellulose occurs during the drying of beans (Mussatto, Carneiro, Silva, Roberto and Teixeira, 2011). Moreover, the application of lignocellulolytic fungi improve the composting process of biomass where the C:N ratio was not at the optimal rate (Hart, Leij, Kinsey and Lynch, 2002).

1.2 Problem Statement

Several potential strains of *Trichoderma* are beneficial features which have been used as a producers of a range important hydrolytic enzymes, as well as a biological control agents (Verma, Brar, Tyagi, Surampally and Valéro, 2007; Sant, Casanova, Segarra, Avilés, Reis and Trillas, 2010). Previously, *Trichoderma* spp. identification is dependent primarily on morphological characters such as growth rate, colony color, size and length of conidia and phialides. Alvindia and Hirooka (2015) claimed that the size of conidia and phialides overlapped between *T. catoptron* and *T. stramenium*, made identification more complicated. Additionally, the morphological and cultural characters are difficult to accurately define species. Gams and Bissett (1998) reported that variations among *Trichoderma* spp. could not be differentiated satisfactorily *via* morphological methods, thus making nomenclature placement uncertain.

DNA sequence analysis became a new paradigm in fungal systematics for *Trichoderma* (Samuels, 2006). The development of molecular tools has enabled the positive identification of any strains. Early stages of molecular research of *Trichoderma* identification rely on the sequencing of ITS regions of rDNA. In addition, a multigene approach using at least two unlinked loci is desirable for the molecular identification of closely related *Trichoderma* spp. Genes of *tef1, rpb2, cal* and act, for example, can be used in combination with ITS regions to reflect

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differences between and within groups of closely related species. Hence, combination of morphological and DNA data will provide at the species level of *Trichoderma* identification. The result from this study stress the importance of combination both morphological and molecular identification tools to describe diversity of *Trichoderma* in paddy fields of Tuaran.

Microbial composting is an effective alternative for the recycling of biomass materials into compost. It promotes sustainable agriculture and environmental protection, as well improving soil's physical, chemical and biological properties (Mylavarapu and Zinati, 2009). Composting process required rapid biodegradation to break down complex lignin, cellulose and starch. *Trichoderma* spp. has the potential to degrade lignocellulosic materials efficiently. Therefore, this study also focused on the screening and evaluating the compatible lignocellulolytic potential among *Trichoderma* spp. for rapid and environmental friendly composting process.

1.3 Hypothesis

Accurate identification of *Trichoderma* species requires both molecular genotyping and phenotypical characterization. Specific *Trichoderma* strains produced vast amount of lignocellulolytic enzymes such as cellulases and hemicellulase.

1.4 Research Objectives

The main focus of this study is to characterize and identify *Trichoderma* spp. isolated from wet paddy field soil samples, Tuaran. The species identification was conducted by using morphological characterization and sequencing analysis of the ITS region, *tef1* and *cal* genes. Moreover, the lignocellulolytic activities of *Trichoderma* spp. were evaluated by screening their degradation towards lignin, cellulose and starch degradation.

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