การพิสูจน์เอกลักษณ์ของราเอนโดไฟต์โดยมัลดิทอฟแมสสเปกโทรเมตรี

นางสาว เบญจพร เทียนสงค์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2549

IDENTIFICATION OF ENDOPHYTIC FUNGI BY MALDI-TOF MASS SPECTROMETRY

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of science
Chulalongkorn University
Academic year 2006

Thesis Title	IDENTIFICATION OF ENDOPHYTIC FUNGI BY MALDI-TOF MASS SPECTROMETRY
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Field of study	Biotechnology
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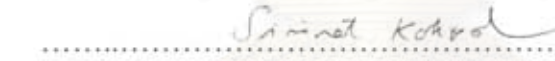

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ในงานวิจัยนี้ ได้นำเทคนิค matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) มาใช้ในการพิสูจน์เอกลักษณ์ของราเอนโดไฟต์ โดยทำการสกัดสารประกอบโปรตีนจากราเอนโดไฟต์ พบว่าราเอนโดไฟต์สามารถผลิตสารประกอบโปรตีนได้หลังจากการบ่มเป็นเวลา 3 วัน จากนั้นนำมาวิเคราะห์ด้วยเทคนิค gel electrophoresis พบว่ามวลโมเลกุลของโปรตีนส่วนใหญ่อยู่ระหว่าง 14.4-97.0 กิโลดาลตัน และเมื่อนำมาวิเคราะห์ด้วยเทคนิค matrix-assisted laser desorption/ ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) พบว่าสเปกตรัมที่ได้จากการวิเคราะห์โปรตีนที่สกัดจากน้ำเลี้ยงเชื้อและเส้นใยของเชื้อราเอนโดไฟต์ มีลักษณะของสเปกตรัมแตกต่างกันในแต่ละสายพันธุ์ของราเอนโดไฟต์ สเปกตรัมของโปรตีนที่แตกต่างกันนั้น จะใช้เป็นโปรตีนเครื่องหมายในการจำแนกราเอนโดไฟต์แต่ละชนิด นอกจากนี้ในงานวิจัยได้พัฒนากระบวนการในการวิเคราะห์ให้ละเอียดมากยิ่งขึ้น โดยทำการศึกษาตรวจวัดมวลโมเลกุลของเปปไทด์ พบว่ามวลโมเลกุลที่ได้จากสเปกตรัมมีความเป็นเอกลักษณ์แตกต่างกันในราเอนโดไฟต์แต่ละชนิด ซึ่งสามารถนำมาใช้ในการระบุชนิดของราเอนโดไฟต์ได้

สาขาวิชา.....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....เบญจพร เทียนสงค์.....

ปีการศึกษา.....2549..... ลายมือชื่ออาจารย์ที่ปรึกษา.....พลกฤษณ์.....

4772354823: MAJOR BIOTECHNOLOGY
KEY WORD: ENDOPHYTIC FUNGI / MALDI-TOF MS / SDS-PAGE / TRYPSIN DIGESTION / PEPTIDES

BENJAPORN THIENSONG: IDENTIFICATION OF ENDOPHYTIC FUNGI BY MALDI-TOF MASS SPECTROMETRY. THESIS ADVISOR: ASSOC. PROF. POLKIT SANGVANICH, Ph.D., 81 pp.

In this research, describes the uses of matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) for rapid identification of endophytic fungi were described. The endophytic fungi were grown in potato dextrose broth and cultures broths were collected at 3 days. Protein molecular weight was measured by SDS-polyacrylamide gel electrophoresis. Almost of major protein bands were presented ranging in molecular weight from 14.4 to 97.0 kDa. Then, the protein was analyzed using MALDI-TOF MS. MALDI mass spectra obtained from the extracellular and intracellular proteins secreted by endophytic fungi showed many differences in mass spectra between the species. Proteins that were specific to each species were also identified. These "marker proteins" may useful for identification and characterization of endophytic fungi species. In addition, the analysis of tryptic peptide mixture can be use for identify the endophytic fungi species. The results indicated good repeatability and the calculation values of statistic shown the similarity values with small standard deviation.

Field of study……Biotechnology…… Student's signature…Benjaporn Thiensong…
Academic year …….2006……..... Advisor's signature…Polkit Sangvanich……..

ACKNOWLEDGEMENTS

I am especially grateful to my advisor, Associate Professor Dr. Polkit Sangvanich for their valuable guidance and assistance throughout my studies and research at Chulalongkorn University. I wish to thank my graduate committee members, Associate Professor Dr. Sirirat Kokphol, Associate Professor Dr. Amorn Petsom, Associate Professor Dr. Nattaya Ngamrojnavanich and Dr. Jittra Piapukiew for their helpful advice and comments.

Additionally, I would like to thank Miss Narumon Sawasdipuksa, Miss Pornpimol Tipthara and Mr. Aphichart Karnchanatat in Protein Unit at Research Centre for Bioorganic Chemistry for their suggestion, helping and teaching me the experimental techniques throughout this work. I wish to thank Miss Srinuan Tansuwan in Bioorganic Unit at Research Center for Bioorganic Chemistry for the supply of endophytic fungus.

I would like to thank the Faculty of Science, Graduate School, Chulalongkorn University for financial support. I am also thanking the Research Centre of Bioorganic Chemistry, Chulalongkorn University for the laboratory facilities. I thank all of my friends, all members in Research Centre for Bioorganic Chemistry, and officers of Biotechnology Program for their friendship and helps during my graduate studies.

Finally, I would like to appreciate my parents for their great support and encouragement throughout my education.

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LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
µmol	micromolar
ACN	Acetonitrile
Bis	*N,N'*-methylenebisacrylamide
BSA	Bovine serum albumin
^{0}C	Degree Celsius
C	Crosslinking factor [%]
HCCA	α-cyano-4-hydroxycinnamic acid
cm	centimeter
Da	Dalton
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
ESI	Electrospray ionization
g	gram
h	hour
IAA	Iodoacetamide
kDA	Kilodalton
L	liter
M	Molar
mA	Milliampare
MALDI	Matrix Assisted Laser Desorption Ionisation
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation/Time of flight
min	Minute
mg	Miligram
mg/ml	Milligram per milliter
ml	Milliter
mm	Millimeter
mM	millimolar

MS	Mass spectrometry
m/z	Mass per charge
nm	Nanometer
PDA	Potato dextrose Agar
PDB	Potato dextrose Broth
rpm	Revolution per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T	Total acrylamide concentration (%)
TEMED	*N*,*N*,*N'*,*N'*-tetramethylenebisacrylamide
TFA	Trifluoroacetic acid
TOF	Time of flight
Tris	Tris (hydroxymethyl)-aminoethane
V	Volt
v/v	Volume by volume
w/v	Weight by volume

CHAPTER I

INTRODUCTION

At present, endophytic fungi are expected to be an important source for new natural bioactive agents because there are several bioactive agents produced by endophytic fungi which can be useful in the pharmaceutical, medical and agricultural industry. Thus, the endophytic fungi have received more attention investigation on physiology, morphology, ecology and genetics etc. Recently, a number of bioactive metabolites which produced by endophytic fungi has been reported. For example, *Paecilomyces* sp. has been isolated from *Taxus mairei, Cepphalataxus fortunei* and *Torreya grandis*, which have antitumor and antifungal activity [1]. *Aspergillus fumigatus* from the leaf of *Cynodon dactylon* produced two new bioactive metabolites, named asperfumoid and asperfumin which were subjected to in vitro bioactive assays against three human pathogenic fungi *Candida albicans, Tricophyton rubrum* and *Aspergillus niger* [2].

The endophyte fungi usually lived asymptomatically within tissue of their host plants [3]. The relationship between the endophyte and its host plant was participating in a mutualistic symbiosis [4]. That is, the plant was thought to provide nutrients to the microbe, while the microbe may produce factors that protected the host plant from attacked by animals, insects or microbes such as the endophytic fungi isolated from *Gaultheria procumbens* L. [5] could be produce new bioactive metabolites which against insect herbivores and may be used as biocontrol agents that they are worthy of further exploration [6].

The identification of endophytic fungi has been carried out by several methods. The basic method is examining their morphology such as characteristic of the mycelium and the spores etc. *Tubercularia* sp. Stain TF5 was isolated from *Taxus mairei* was identified by the mechanism of spore production and the characteristic of the spores [7]. However, there are a species that share strong morphological similarities to each other and cannot be identified based on their morphological characteristics alone.

Another method is molecular biology that developed to reduce the morphological confusion. These methods include DNA hybridization, restriction fragment length polymorphism (RFLP), and 16s rDNA gene sequencing [8]. In the 2002s, Tanaka *et al*. were identical the endophytic fungi that isolated from *Abelmoschus* sp. by the sequence analysis. From this technique, this endophytic fungus was found to be similar to *Collectrotrichum gloeosporiodes* and *C. dematium.* According the molecular biology data, they identified this endophytic fungus as *Collectrotrichum* sp. [9].

However, the molecular technique is more complicated steps for analysis and takes a long times. Therefore, in this research have used mass spectrometry was used to identify the endophytic fungi, which the advantages including a more rapid analysis time and low sample volume requirement.

Mass spectrometry is an analytical technique for establishment of the molecular weight and the structure of organic or inorganic compounds, which measured from the mass to charge (m/z) ratio of ions. This technique allows powerful tool and very high sensitivity that could be used to identify biological macromolecules such as proteins [10, 11]. From the properties, these techniques have been used to identify the endophytic fungi due to each species of endophytic fungi expressed difference of proteins.

Previous research, has been reported on the identification of protein of whole bacterial cell, with matrix assisted laser desorption ionization (MALDI) [12]. Proteins are mixed in a matrix and transforms with the laser energy into excitation energy for the proteins. At the same time, a minute amount of the matrix rapidly heats and analyte molecules into gas phase. Accompanying the matrix assisted laser desorption ionization technique is time of flight mass spectrometer (TOF/MS), which is a form of mass spectrometer that utilizes differences in time of flight due to size differences of ionized samples. The lighter ions travel faster and reach the detector earlier than those of the heavier ones. Therefore, the ions can be isolated by size differences of ionized samples.

In this study, mass spectrometry will be used to identify the endophytic fungi using proteomic databases and develop the method for the identification of endophytic fungi.

Objectives:

(1) To develop a method for the identification of endophytic fungi by mass spectrometry.

(2) To establish a library of mass spectral fingerprints of endophytic fungi.

(3) To evaluate the reproducibility of this mass spectrometry.

CHAPTER II

THEORETICAL AND LITERATURE REVIEW

Theoretical background

2.1 Endophytic fungi

Most plants are important host for one or more endophytic microorganism. The relationship between the endophyte and its host plant is participating in a mutualistic symbiosis. The endophyte is a bacterial or fungal microorganism, which spends the whole or part of its life cycle colonizing intercellularly or intracellularly inside the healthy tissues of the host plant (Figure 2.1), typically causing no apparent symptoms of disease [13].

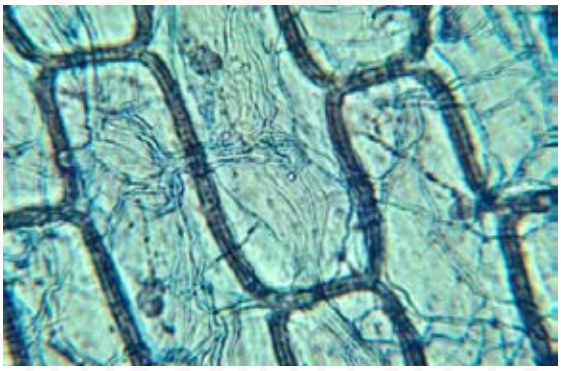


Figure 2.1 Endophytic fungal hyphae between the cells of tall fescue stems

Currently, the endophytes are also much renewed attention is now being paid to the chemistry and bioactivity of endophyte metabolites, and to endophytic biodiversity and related ecological functions.

In the 1904s, endophytic fungus was the first recovered in darnel plants [13]. They have attracted great attention in the past few decades for two main reasons [14, 15].

First, the endophytic fungi are found in all plant, are extremely abundant and are often very diverse. Most of these endophytic fungi form internal localized infections in foliage, roots, stems and bark, and are horizontally transmitted via spores. A much smaller fraction, mostly found in grasses and form systemic infections in above-ground tissues. Some of these are vertically transmitted via hyphae growing into seeds.

Second, endophytic fungi may produce bioactive metabolites, or otherwise alter host physiology and morphology. Endophyte metabolites are thought to benefit their woody plant hosts as ‘inducible defenses’ against insect herbivores and their grass hosts as ‘acquired plant defenses’ against both vertebrate and invertebrate herbivores. Endophytes may also alter other physiological, developmental or morphological properties of host plants such that competitive abilities are enhanced, especially in stressful environments.

2.1.1 Groups of endophytic fungi [3]

Endophytic fungi are often divided into two groups:

(1) Clavicipitaceous endophyte is endophyte of grass that often confer resistance to herbivory and pathogens, which divided into family clavicipitaceae.

(2) Non-Clavicipitaceous endophyte representing all other fungal groups on diverse trees and shrub hosts. The endophytes in these groups such as fungi in sub-division Ascomycotina, Deuteromycotina, Zygomycotina and Basidiomycotina.

2.1.2 Biodivesity of endophytic fungi [13]

Almost all vascular plant species were found to harbor endophytic bacterial or fungi. Moreover, the colonization of endophytes has also been found in marine plants, red and brown algae, mosses and ferns. Commonly, several to hundreds of endophyte species can be isolated from a single plant. Endophytic fungi are present in most plant parts, especially the leaves that the tissue is apparently healthy. Endophytes can be isolated from mildly surface sterilized plant tissues and cultivated on nutrient agar (Figure 2.2). The environmental conditions under which the host is growing also affect the endophytic fungi population, and the endophytic fungi profile may be more diversified in tropical areas. From the investigation, genotypic diversity has been observed in single endophyte species originating from conifers and grasses. Accordingly, endophytes are presumably ubiquitous in the plant kingdom with the population being dependent on host species and location.

Figure 2.2 Endophytic mycelia growing out from cut areas of surface sterilized plant segments.

2.1.3 Origin and evolution [13]

Some phytopathogens in the environment are of endophyte origins. Many innocuous fungal endophytes are quiescent phytopathogens which may cause infectious symptoms when the host plant is aged or stressed. On the other hand, during the long co-evolution of the phytopathogens and its host plant, an endophytic

mutant may result from balanced antagonism or gene mutation. Dual cultures of the host calli and endophytes demonstrated that both the endophytes and the host calli excrete metabolites toxic to each other. Further investigation led to the development of a hypothesis that the endophytes host interaction could be a balanced pathogen host antagonism. Freeman (1993) and Rodriguez (1993) found that a naturally occurring nonpathogenic endophytic mutant developed from the mutation of a single locus in the genome of the wild type *Collectotrichum magna*, a pathogen causing anthracnose in cucurbit plants. This mutant is able to grow systemically inside the host plant without pathogenic symptoms, but retaining wild type levels of *in vitro* sporulation, spore adhesion, appressoria formation, infection and host specificity.

2.2 Identification of microorganisms methods [34]

Classically, morphology physiology and biochemistry are common methods used to identification of microorganisms. Morphological and physiological are the basic methods for identified microorganisms which these methods examination was performed by characteristic of the mycelium and the spores and the mechanism of mycelium and spore production. In addition, the identification of microorganisms is often uncertain a similar morphological. The use of these methods requires the experts for identification of microorganisms. For biochemical, involves the characterization of phenotypes of microorganisms. However, there a number of species that share strong phenotypic similarities to each other and cannot be identified based on their phenotypic characteristics alone.

Modern in identification of microorganisms methods are molecular biology and protein identification. Molecular techniques were developed to reduce the phenotypic confusion. These techniques include DNA hybridization, restriction fragment length polymorphism (RFLP), and 16s rDNA gene sequencing. These techniques revealed that a number of phenotypically similar strains were indeed different at genome level.

For the past thirty five years, a number of identification of proteins has been developed to quickly differentiate microorganisms. Protein expressed in microorganisms can be used as biomarkers for microorganism identification. Each protein in microorganisms has its own unique sequence, which defines its biological function and chemical behavior.

2.3 Protein identification [18]

Traditionally, antibodies and photoaffinity labeling are methods used to identification of proteins, and subsequent Edman sequence analysis for the characterization of proteins. Antibodies are used for Western blots, and other immunology-based methods such as enzyme-linked immunoassay (ELISA) and radio immunoassay (RIA). The use of antibodies, although rapid and sensitive, requires the ready availability of an extensive library of suitable antibodies. In addition, the identification of proteins is often uncertain a similar structure. Similarly, photoaffinity labeling followed by autoradiography is also very sensitive, but requires the use of hazardous radioactive labels. For Edman sequencing, involves the sequential degradation of peptide from the N terminus, and the identification of the released amino acids one at time. The Edman technique has several limitations. It is requires a long time and a large amount of sample.

In molecular biology techniques have made it possible to derive the amino acid sequence of a protein from a DNA sequence. Although this technique requires less effort and is more sensitive than the Edman techniques, errors in DNA-derived sequence may occur because a protein expressed by recombinant DNA techniques is not necessarily identical to the native protein.

Advances in protein identification techniques are determination of the molecular weight of proteins and peptides which the molecular weight of proteins is a unique characteristic of each protein of microorganisms. Electrophoresis and mass spectrometry are widely use to the determination of the molecular weight of proteins or peptides. These techniques will be described in detail later.

2.4 Electrophoresis

Electrophoresis is an analytical method frequently used in molecular biology, biochemical research, protein chemistry, pharmacology, forensic medicine, clinical investigations and food control. It is applied for the separation and characterization of proteins, nucleic acids and subcellular-sized particles like viruses and small organelles. Its principle is that the migration of charged molecules in solution in response to an electric field. Their rate of migration depends on the strength of the field, on the charge, size and shape of the molecules and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used analytically to study the properties of a single charge species, and as a separation technique [16, 17].

2.4.1 Gel electrophoresis

Gel electrophoresis is a method that separates macromolecules either nucleic acids or proteins on the basis of size, electric charge, and other physical properties. When a macromolecules such as proteins or nucleic acids, is mixed in a buffer solution and applied to a gel. The electrical current from one electrode repels the molecules while the other electrode simultaneously attracts the molecules. The frictional force of the gel material acts as a "molecular sieve" separating the molecules by size. During electrophoresis, macromolecules are forced to move through the pores when the electrical current is applied. After staining, the separated macromolecules in each lane can be seen in a series of bands spread from one end of the gel to the other [16].

There are two types of buffer systems used in gel electrophoresis: continuous and discontinuous. A continuous system has a single separating gel and uses the same buffer in the tanks and the gel. In discontinuous system, first developed by Ornstein (1964) and Davis (1964), a nonrestrictive large pore gel, called a stacking gel, is layered on top of a separating gel called a resolving gel (Figure2.3). Each gel is made with a different buffer, and the tank buffers are different from the gel buffers. The resolution obtained in a discontinuous system is much greater than that obtained with a continuous system [17].

The discontinuous Laemmli system (1970), a denaturing modification of Ornstein (1964) and Davis (1964), is the most widely used system for research protein electrophoresis today. The resolution in a Laemmli gel is excellent because the treated peptides are concentrated in a stacking zone before entering the separating gel [17].

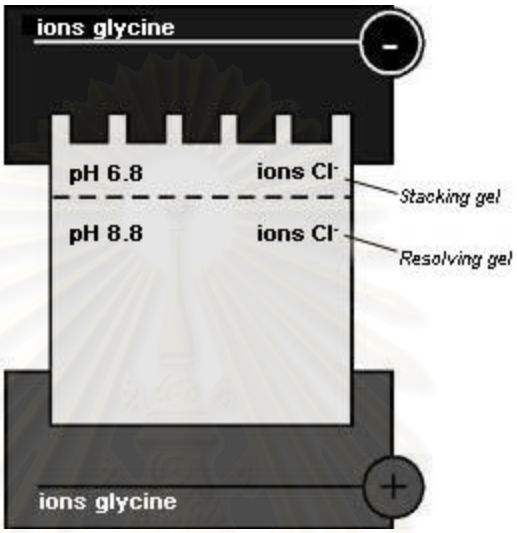


Figure 2.3 Electrophoresis through the stacking (pH 6.8) and resolving (pH 8.8) gels

Generally the sample is run in a support matrix such as paper, cellulose acetate, starch gel, agarose and polyacrylamide gel. A very common support matrix for separating macromolecules by electrophoresis uses agarose and polyacrylamide gel. For both polymers, the degree of cross linking of the matrix, and hence the gel pore size can be varied according to size range required for fractionating the macromolecules under investigation. For proteins, most commonly used gel is polyacrylamide and sodium dodecyl sulfate (SDS) to denature the proteins. The technique is called sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [16, 17].

2.4.2 Sodium dodecyl sulfate (SDS)

Sodium dodecyl sulfate (SDS) is an anionic detergent, meaning that when dissolved its molecules have a net negative charge within a wide pH range. A polypeptide chain binds amounts of SDS in proportion to its relative molecular mass. The negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted toward an anode (positive charged electrode) in electric field.

2.4.3 Polyacrylamide gel [20]

Polyacrylamide gels were first used for electrophoresis by Raymond and Weintraub (1959). They are chemically inert and mechanically stable. By chemical co-polymerization of acrylamide monomers with a cross-linking reagent usually N,N'- methylenebisacrylamide (Figure 2.4). The polymerization of acrylamide is initiated by addition of ammonium persulfated and the base *N,N,N',N"*-tetramethylenediamine (TEMED). TEMED catalyzes the composition of persulfate ion to give a free radical.

The pore size can be exactly and reproducibly controlled by the total acrylamide concentration *T* and the degree of cross linking *C*:

$$T = \frac{(a+b) \times 100}{V} \ [\%] \quad \text{and} \quad C = \frac{b \times 100}{a+b} \ [\%]$$

a is the mass of acrylamide in g,

b the mass of methylenebisacrylamide in g, and

V the volume in mL

When *C* remains constant and *T* increases, the pore size decreases. The range of pore size varies from 4% *T* to 20% *T*. Gel with higher % *T* has small pore sizes and more restrictive so it favors the movement of smaller proteins with little or no movement of large proteins.

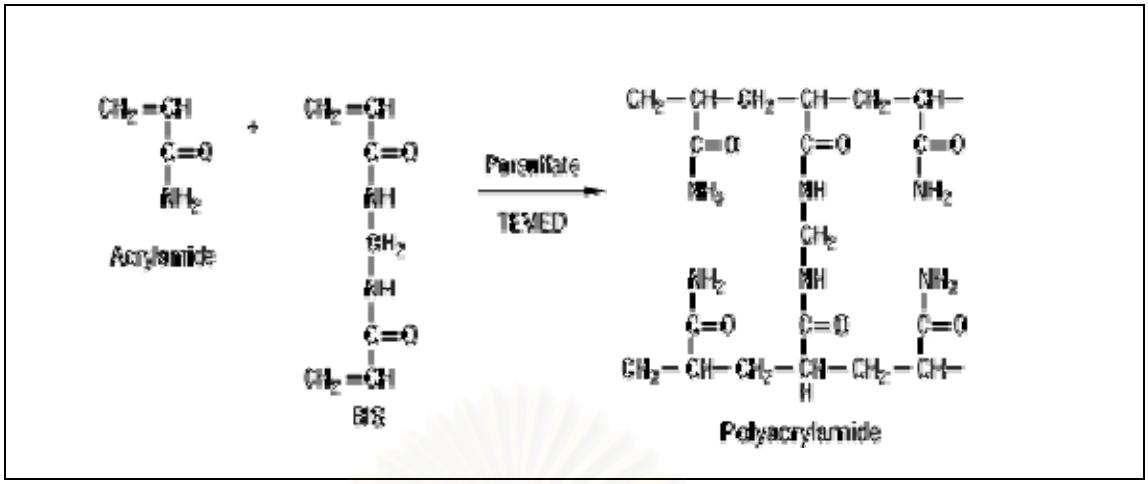


Figure 2.4 The polymerization reaction of acrylamide and methylenebisacrylamide

Polymerization should take place under an inert atmosphere since oxygen can act as a free radical trap. The polymerization is temperature dependent: to prevent incomplete polymerization the temperature should be maintained above 20^0C.

Electrophoresis is usually carried out with a constant polymerization concentration in the gel, chosen to correspond to the required molecular weight range (Table 2.1). To extend this range in a single electrophoresis experiment, gradient gels, formed with a gradient mixer, can be used. A linear gradient of 5-20 % total acrylamide concentration allows proteins in the molecular weight range 15-200 kDa to be separated.

Table 2.1 Protein separation range in polyacrylamide gel electrophoresis

Gel concentration [%(w/v)]	Molecular weight range [kDa]
3-5	> 100
5-12	20-150
10-15	10-80
> 15	< 15

2.5 Mass spectrometry [18,21,33]

Mass spectrometry is an analytical technique for measuring the molecular mass of a sample, studying molecular structural elucidation of organic compounds and probing the fundamental principles of chemistry. In proteins analysis, mass spectrometry is useful for the structure elucidation of proteins and for peptide sequencing which can be generated using certain types of mass spectrometers, usually those with multiple analyzers which are known as tandem mass spectrometer.

The mass spectrometer is an instrument that serves for establishment of the molecular weight and the structure of organic or inorganic compounds. The three essential functions of a mass spectrometer and the associated components are: (Figure 2.5)

(1) an ion source where the sample of compounds are ionized, usually to cations by loss of an electron.

(2) an mass analyzer which separates gas-phase ions according to their mass to charge (m/z) ratio.

(3) a detector which give the ion intensities, and the results are displayed on a chart.

Figure 2.5 General mass spectrometry scheme

Principles

The principle of mass spectrometric analysis for organic compounds. The sample is volatilized within the spectrometer that the sample molecule is ionized to gas-phase ions. These ions are represented as positive charges and referred to as the molecular ion ($M^{\bullet+}$):

$$M + e^- \longrightarrow M^{\bullet+} + 2e^-$$

Molecular ion

This molecular ion normally undergoes fragmentations. Because it is a radical cation with an odd number of electrons, it can fragment to give either a radical and an ion with an even number of electrons, or a molecule and a new radical cation. Let us stress the important difference between these two types of ions and the need to write them correctly:

$$M^{\bullet+} \begin{cases} \nearrow & EE^+ + R^{\bullet} \\ & \text{even ion} \quad \text{radical} \\ \searrow & OE^{\bullet+} + N \\ & \text{odd ion} \quad \text{neutral molecule} \end{cases}$$

These two types of ions have different chemical properties. Each primary product ion derived from the molecular ion can, in turn, undergo fragmentation, and so on. All these ions are extracted into the analyzer region of the mass spectrometer where they are separated according to their mass (m) -to- charge (z) ratios (m/z). Generally, most of the ions have a charge corresponding to the loss of only one electron. The separated ions are detected and this signal sent to a data system where the m/z ratio is stored together with their relative abundance for presentation in the format of a m/z spectrum.

Because of ions are very reactive and short lived, their formation and manipulation must be conducted in a vacuum. Thus, the analyzer and detector of mass spectrometer, and often the ionization source too, are maintained under high vacuum (10^{-5} – 10^{-8} torr) to give the ions a reasonable chance of traveling from one end of the instrument to the other without any hindrance from air molecule.

2.5.1 Methods of sample ionization

Many ionization methods are available and each has its own advantages and disadvantages which the choice is depending on the physical state, the volatility and thermal stability of the sample. The ionization methods used for the majority of biomolecules analyses are Electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI). These methods allow the high-precision analysis of biomolecules of very high molecular weight. The electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) will be described in detail later.

2.5.2 Analysis and separation of sample ions

The main function of the mass analyzer is to separate, the ions formed in the ionization source of the mass spectrometer according to their mass-to-charge ratios. There are a number of mass analyzers currently available, the better known of which include quadrupoles, time-of-flight mass analyzer, magnetic sectors and both fourier transform and quadrupoles ion traps. These mass analyzers have different features, including the m/z range that can be covered, the mass accuracy, and the achievable resolution. The compatibility of different analyzers with different ionization methods varies. Mass analyzers are widely used time-of-flight mass analyzer and quadrupoles mass analyzer.

2.5.3 Detection and recording of sample ions

The detector monitors the ion current, amplifies it and the signal is then transmitted to the data system where it is recorded in the form of mass spectra. The m/z values of the ions are plotted against their intensities to show the number of components in the sample, the molecular mass of each component, and the relative abundance of the various components in the sample. The types of detector is supplied to suit the type of analyzer, the most common ones are the photomultiplies, the electron multiplier and the micro-channel plate detectors.

2.5.4 Electrospray ionization (ESI)

Electrospray ionization is one of the atmospheric pressure ionization (API) techniques and is widely use to the analysis of polar biomolecules such as proteins, peptides and glucose etc. This technique allows very high sensitivity to be reached and is easy to couple to high-performance liquid chromatography (HPLC).

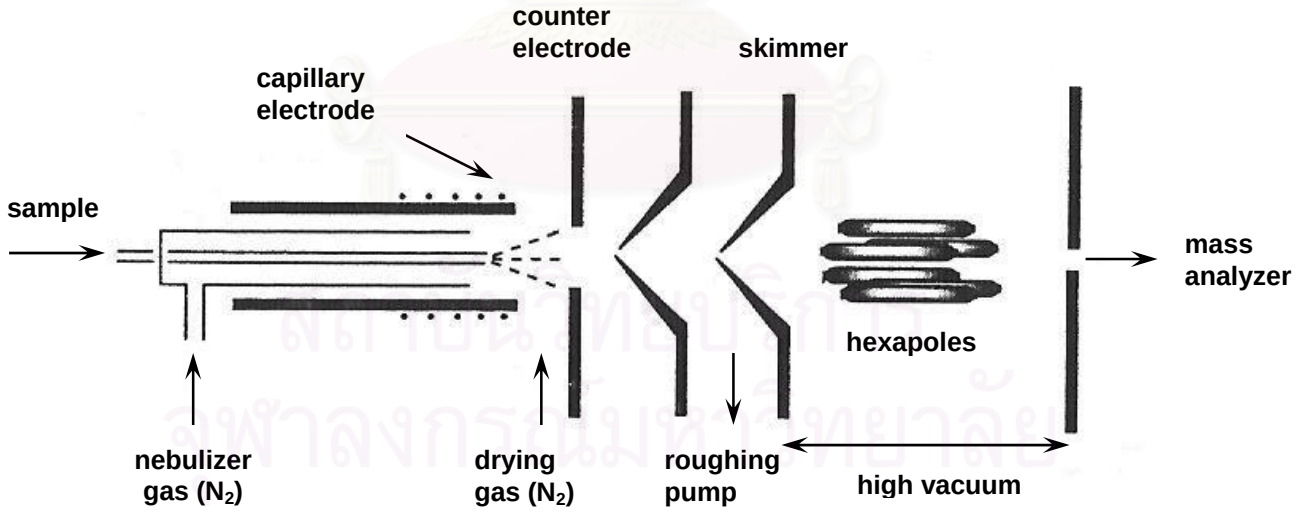


Figure 2.6 Schematic of electrospray ionization source

An electrospray is produced by applying a strong electric field, to a liquid passing through a capillary tube with a flow rate of between 1-10 μl min^{-1}, under atmospheric pressure. The electric field is obtained by applying a potential difference of 3-6 kV between this capillary and the counter-electrode, separated by 0.3-2 cm (Figure 2.6). When, the sample emerging from the capillary tip is dispersed into an aerosol of highly charged droplets. The charged droplets are evaluated due to solvent evaporation and droplet fission caused by coulombic repulsion of the charges on the droplets. When the electric field on their surface becomes larges enough, desorption of ions from the surface occurs. Charges in excess accumulate at the surface of the droplet. Eventually charged ions, are released from the droplets, some of which pass through an analyzer of mass spectrometer, which under high vacuum (Figure 2.7).

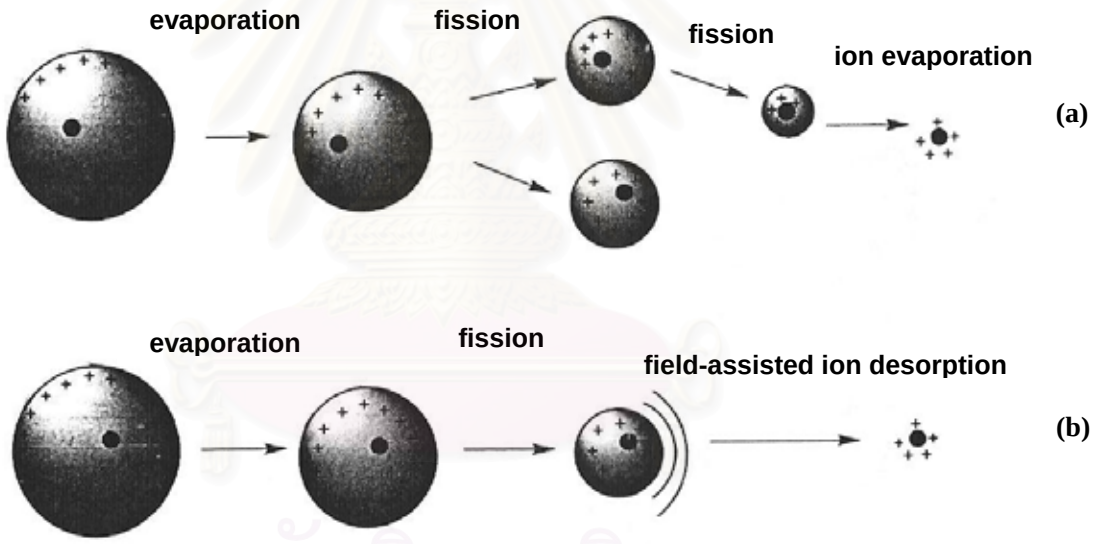


Figure 2.7 Schematic of ion formation during electrospray: (a) charge residual model; (b) ion desorption model.

Electrospray has important characteristics; it is able to produce multiply charged ions from larges molecules. Obtaining multiply charged ions is advantageous because it improves the sensitivity at the detector and it allows the analysis of high molecular weight molecules using analyzers with a weak nominal mass limit. Indeed, the technical characteristics of mass spectrometers are such that the value being measured is not mass, but the mass-to-charge ratio (m/z). This technique allowed the determination of the molecular mass of proteins above 130 kDa with a detection limit of about 1 pmol using a quadrupole analyzer. Thus the m/z value obtained from ESI/MS was able to determine a molecular ion with mass M (Da) by the following equation:

$$z_1 m_1 = M + z_1 m_p \quad \text{..... (1)}$$

$$m_2 (z_1 - j) = M + (z_1 - j)\, m_p \quad \text{..... (2)}$$

These two equations lead to:

$$z_1 = \frac{j (m_2 - m_p)}{(m_2 - m_1)} \quad \text{and} \quad M = z_1 (m_1 - m_p)$$

In the case of negative multiply charged ions, analogous equations lead to:

$$z_1 = \frac{j (m_2 + m_p)}{(m_2 - m_1)} \quad \text{and} \quad M = z_1 (m_1 + m_p)$$

where z = the number of charges

m = the mass-to-charge ratio

m_p = the mass of the proton

M = the molecular mass of sample

2.5.5 Quadrupole mass analyzer

The quadrupole mass analyze consists of four metal rods (Figure 2.8). The rods must be accurately aligned parallel that are arranged symmetrically in a square array.

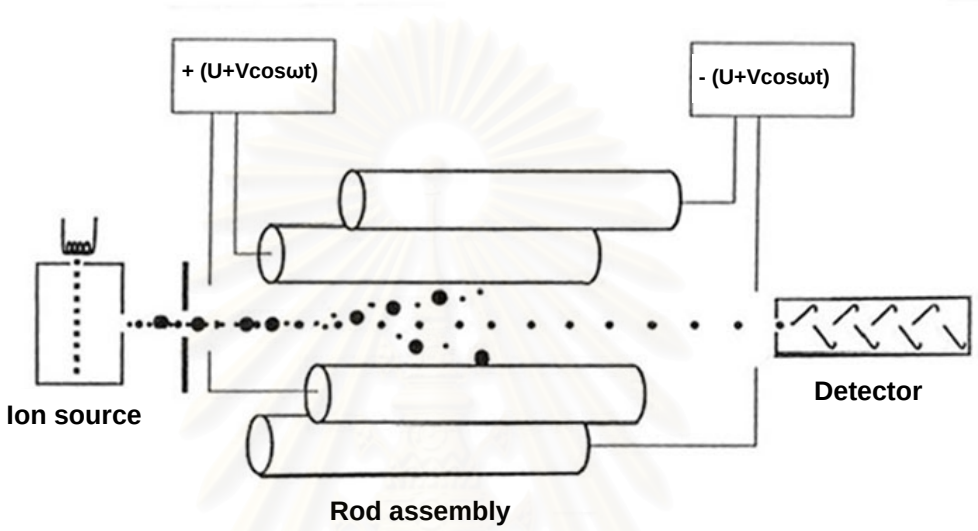


Figure 2.8 Schematic of quadrupole rods

Quadrupoles are dynamic mass analyzers, which the mass separation in this instrument is accomplished by using electric fields. This mass analyzer connected to a direct current (DC) voltage and a radio frequency (RF) potential. Combined DC and RF potential on the quadrupole rods that are produced the electric fields. The electric fields are made the ions to have stable trajectory, and pass to the detector. All other ions have unstable trajectory will collide with the quadrupole rods and the ions are lost.

2.5.6 Matrix assisted laser desorption ionization (MALDI)

Matrix assisted laser desorption ionization (MALDI), is a technique that makes possible the ionization of biological macromolecules such as proteins that are difficult to be ionized because they are easily decomposed. Accompanying the matrix assisted laser desorption ionization technique is time of flight mass spectrometer (TOF/MS), which is a form of mass spectrometer that utilizes differences in time of flight due to size differences of ionized samples (Figure 2.9).

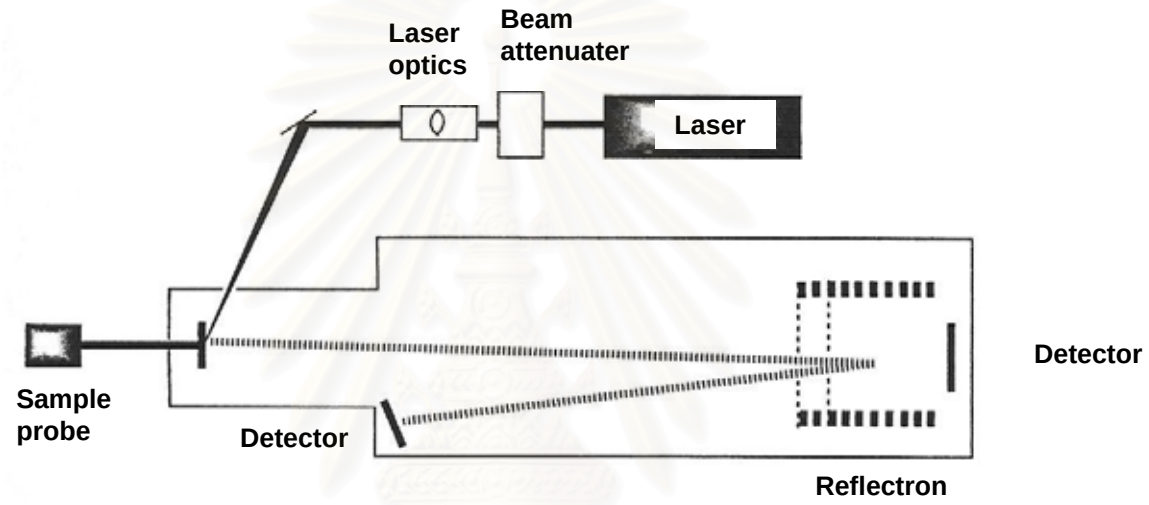


Figure 2.9 A schematic of MALDI-TOF MS

Samples that are analyzed by MALDI (matrix assisted laser desorption ionization) are mixed in a solvent containing small organic molecules in solution, called a matrix, and has a strong absorption at the laser wavelength. The matrix transforms the laser energy into excitation energy for the sample. At the same time, a minute amount of the matrix rapidly heats and analyte molecules into gas phase. Ionization reactions can occur at any time during energy transfer and become ionized via gas-phase proton transfer reactions (Figure 2.10). Most commercially available MALDI mass spectrometers now have a pulse nitrogen laser of wavelength 337 nm.

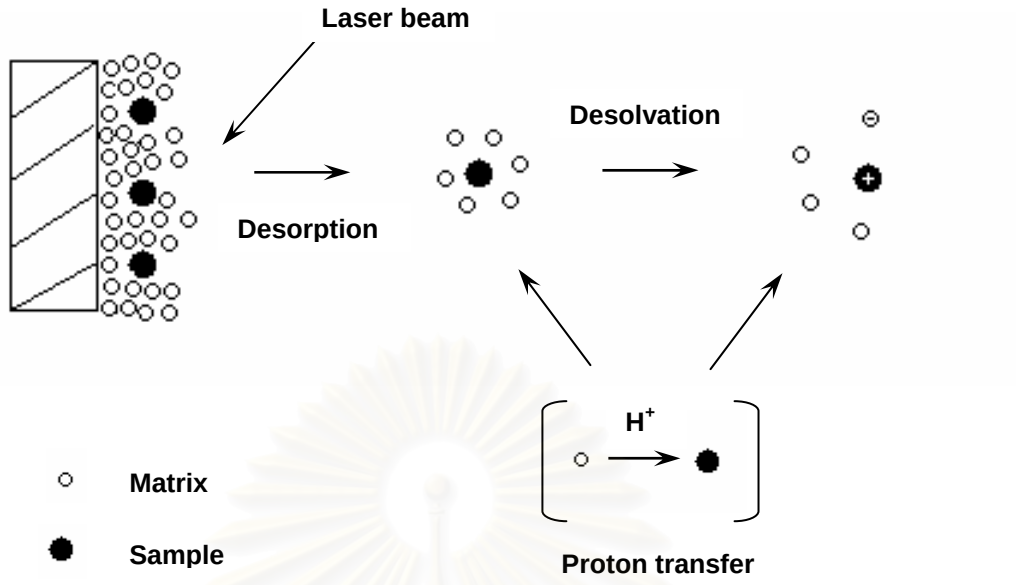


Figure 2.10 Diagram of the principle of MALDI

The MALDI process is more sensitive than other laser ionization techniques. The matrix also serves to minimize sample damage from laser pulse by absorbing most of the incident energy and increase the efficiency of energy transfer from the laser to the analyte. Thus the sensitivity is highly increased. The technique of MALDI mass spectrometry has become a powerful analytical tool for the picomoles of proteins with molecular mass up to 300,000 Da.

Matrix selection and optimization of the sample preparation protocol are the most important steps in the analysis. The MALDI matrix selection is based on the laser wavelength used and the class of compound analyzed. Requirements of MALDI matrices are strong absorbance at the laser wavelength, low enough mass to be sublimable, vacuum stability and lack of chemical reactivity. Some commonly used matrices, along with the wavelengths at which they are used, the solvents in which they can be dissolved, and fields of their applications, are listed in Table 2.2.

Table 2.2 The matrices used in MALDI

Matrix	Mass (Da)	Solvents	Laser λ (nm)	Applications
2,5-Dihydroxybenzoic acid (DHB)	154	ACN[a], water, methanol, acetone, chloroform	266, 337, 355	Oligosaccharides, peptides, nucleotides, oligonucleotides
Cinnamic acid	148	ACN, water	337	General
α-Cyano-4-hydroxycinnamic acid	189	ACN, water, acetone, ethanol	337, 355	Peptides, lipids, nucleotides
3,5-dimethoxy-4- hydroxycinnamic acid (Sinapinic acid)	224	ACN, water, acetone, chloroform	266, 337, 355	Peptides, lipids, proteins
4-hydroxy-3-methoxycinnamic acid (Ferrlulic acid)	194	ACN, water, propanol	266, 337, 355	Proteins
3-Hydroxypicolinic acid (HPA)	139	Ethanol	377, 355	Oligonucleotides
3-Amino-4-hydroxybenzoic acid	153	ACN, water	337	Oligonucleotides
2,4,6-Trihydroxyacetophenone	168	ACN, water	337, 355	Oligonucleotides

[a] ACN = acetronitrile. The laser light of 337 nm is produced with a nitrogen laser, and 266 and 355 nm, with frequency-quadrupoled and –tripled Nd : YAG laser

2.5.7 Time of flight mass analyzer (TOF)

A time of flight mass spectrometer likely as a velocity spectrometer, in which ions are separated on the basis of their velocity differences. In a time of flight mass spectrometer a pulse of ions is created from the sample, which under a high vacuum (10^{-6}-10^{-8} mbar). An electric field accelerates ions into an evacuated chamber, called a field-free drift region. All ions entering the field-free drift region have a fixed kinetic energy is therefore described by:

$$zV = \tfrac{1}{2} m\upsilon^2 = \tfrac{1}{2} m(L/t)^2$$

where:

z = ion charge

V = electric field voltage

m = ion mass

υ = velocity through the drift region

L = length of drift region

t = time of flight through drift region

Mass-to-charge ratios are determined by measuring the time that ions takes to move through a field free region between the source and the detector. The lighter ions travel faster and reach the detector placed at the end of the flight tube earlier than do the heavier ones (Figure 2.11). In order to convert the time spectrum into mass spectrum, their mass-to-charge ratio can be calculated from their flight time after calibration of the analyzer using compounds with known mass ions.

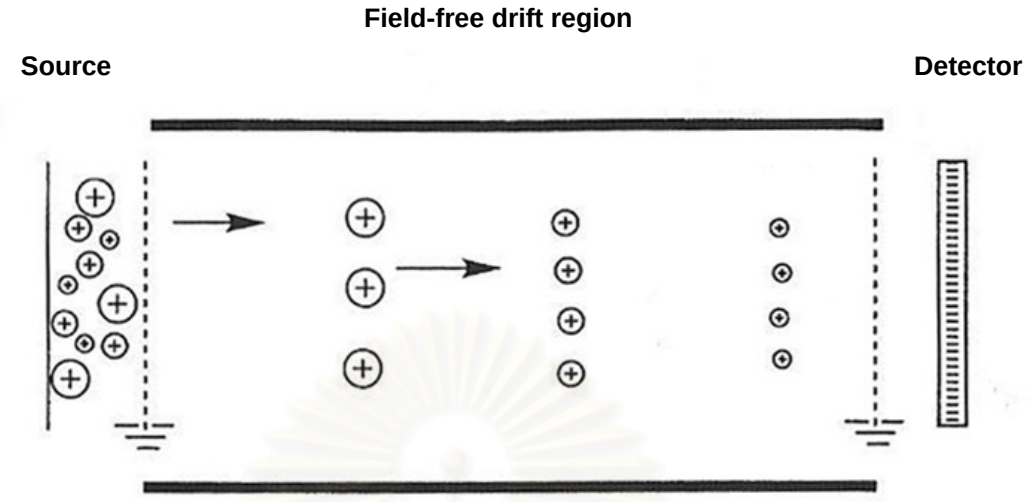


Figure 2.11 Principle of the mass separation by a time-of-flight mass analyzer

For the time of flight mass spectrometer can be increased resolution by the use of an ion mirror (electrostatic reflectron) at the end of the flight tube and by delayed ion extraction out of the ion source (Figure 2.12). The reflectron has an applied voltage higher than that of the accelerating voltage in the ion source, resulting in a slowing down of incoming ions, and finally a reversion of their flight path to the second detector.

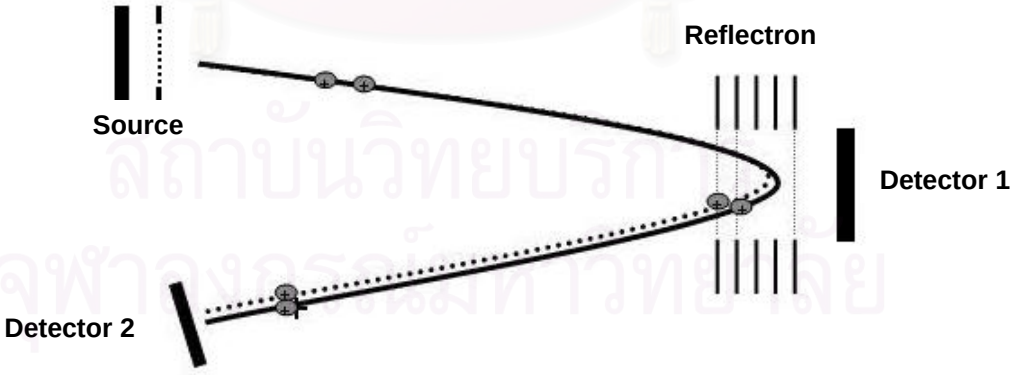


Figure 2.12 MALDI-TOF mass spectrometer operating in reflectron mode

2.6 Literature reviews [22-33]

Form literature survey, there are a number of reports on the identification of microorganisms by mass spectrometry. Most of these studies involve identify and characterize bacteria and viruses. In previously, the ionization of bacterial extracts under fast-atom bombardment (FAB), electrospray ionization (ESI), and sample pyrolysis, followed by mass spectrometry. For example, in 1993 Magee *et al.* reported characterization and taxonomy of *Aeromonas* sp. by pyrolysis mass spectrometry [22].

More recently, the used of whole cell MALDI-MS has been used to rapidly differentiate microorganisms. The MALDI-MS approach generates unique *m/z* signatures for different microorganisms due to inherent differences in the cellular proteins expressed by the microbiological species. Species from the genera *Escherichia* [23], *Helicobacter* [24], *Campylobacter* [24] and *Pseudomonas* [25] have been characterized by MALDI-MS. Pineda et al. and Demirev et al. have successfully identified species of *Helicobacter pylori* using MALDI-MS and internet-accessible proteome databases [27]. The advantages of MALDI-MS over traditional characterization methods include a more rapid analysis time, < 5 min/sample (which reduces the possibility of contamination), low sample volume requirement (< 1.5 µl), and high-throughput with automation.

The identification of fungi species by MALDI-MS approach have been used to identify fungal species, *Saccharomyces cerevisiae* was the first eukaryotic for researched, that is a unicellular eukaryote. However, there is no reporting of the use this technique to identified complex organisms. Thus, these studies aimed to identify endophytic fungi by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS).

CHAPTER III

EXPERIMENTAL

3.1 Material

3.1.1 Endophytic fungi

The endophytic fungal was obtained from the Research Centre for Bioorganic Chemistry (RCBC), Chulalongkorn University, Thailand. There were 9 species of endophytic fungi in this study including *Xyralia* sp., *Bipolaris* sp., Endophytic fungus sp. 00045 5 isolated from oat root, Endophytic fungus isolated J48 isolated from coffee leaves, *Mycoleptodicus* sp., *Emericella* sp., *Glomerella* sp., *Alternaria* sp. and *Nodulispolium* sp. Colonial morphology of the endophytic fungi is shown in Figure3.1. Stock cultures were maintained on potato dextrose agar (PDA) at room temperature (30-35^{0}C) and were transferred to new PDA every 6-7 weeks.

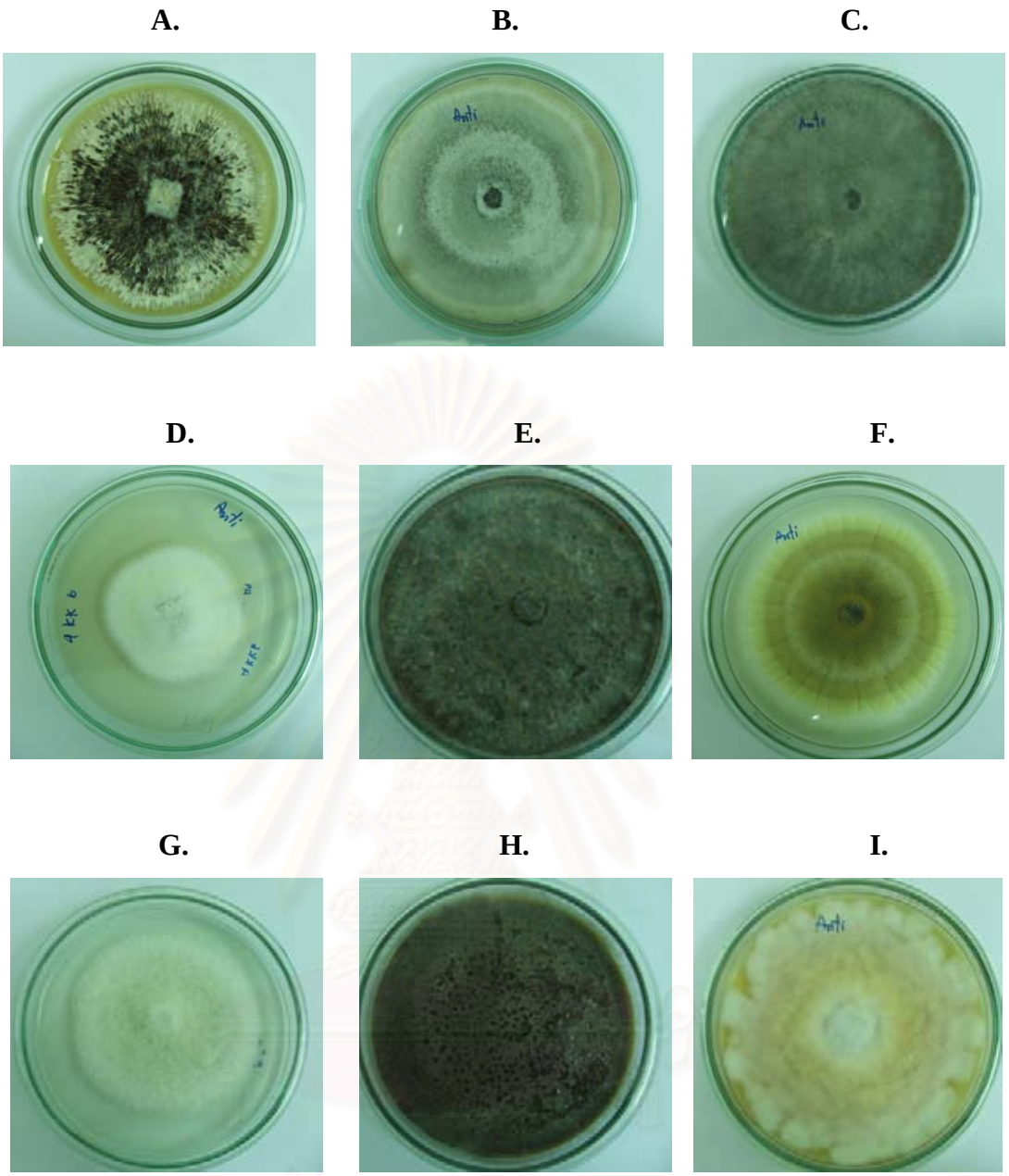


Figure 3.1 Colonial morphology characteristic of endophytic fungi on PDA media after cultivation for 2 weeks at room temperature: A) *Xylaria* sp., B) *Bipolaris* sp., C) Endophytic fungus sp. 00045 5 isolated from oat root, D) Endophytic fungus sp. J48 isolated from coffee leaves, E) *Mycoleptodicus* sp., F)*Emericella* sp., G) *Glomerella* sp., H) *Alternaria* sp. and I) *Nodulispolium* sp.

3.1.2 Apparatus and Instruments

Autoclave: Isuzu seisakusho co., LTD, Japan

Autopipette: Pipetman, Gilson, France

Eppendroff: Axygen Scientific, U.S.A

Freeze dryer: Labconco, U.S.A

Hot air oven: Memmert, Germany Rotary Shaker: 200 rpm

Laminar flow: SAFETY LAB, Asian Chemical and Engineering co., LTD, Thailand

Matrix Assisted Laser Desorption Ionization-Time of Flight Mass spectrometer: BIFLEX, Bruker, Germany

Microcentrifuge: Biofuge pico Heraeus, Kendro, Germany

Microtrap desalting cartridg: Michrom Bioresource

Orbital Shaker: Kika-Werke GMBH&Co., Germany

Peptide Trap: Michrom Bioresource, Germany

pH meter: Denver Instrument, U.S.A

Pipette tips: Bioline, U.S.A

Power Supply: EPS 301, Amersham pharmacia biotech, Sweden

Refrigarated centrifuge: Hettich, Germany

SDS-PAGE: Hoefer^{TM} mini VE (minivertical), 8×9 cm gels, Amersham pharmacia biotech, Sweden

Shaker 200 rpm

Spectrophotometer: TECAN, Austria

Speed vacuum centrifuge: Heto-Holten, Denmark

Sonicator: BHA-1000, Branson, U.S.A

Vortex mixer: Vortex-Genie2, Scientific Industries, U.S.A

Water Bath Shaking: Memmert, Germany

3.1.3 Chemicals and Reagents

Acetic acid: Merck Ag Darmstadt, Germany
Acetone: Merck Ag Darmstadt, Germany
Acetonitrile: Merck Ag Darmstadt, Germany
Acrylamide PAGE: Plusone pharmacia biotech, Sweden
Acrylamide: Amersham pharmacia biotech, Sweden
Ammonium persulfate: Plusone pharmacia biotech, Sweden
Bromophenol Blue: USB, U.S.A
Bovine serum albumin: Sigma, St. Louis, MO, U.S.A
Coomassie brilliant blue: USB, U.S.A
Ethanol: Merck Ag Darmstadt, Germany
Formic acid: Merck Ag Darmstadt, Germany
Glycine: USB, U.S.A
Methanol: Merck Ag Darmstadt, Germany
N, N'- methylenebisacrylamide; Plusone pharmacia biotech, Sweden
Phosphoric acid: J.T. Baker, U.S.A
Potato dextrose broth: Acumedia Manufacturers Inc., U.S.A
SDS (Sodium Dodecyl Sulfate): USB, U.S.A
Trichloroacetic acid: BHD, VWR International Ltd., England
Trifluoroacetic acid: Fluka, Germany
Tris: USB, U.S.A
α-Cyano-4-hydroxycinnamic acid: Sigma Chemical, U.S.A

3.1.4 Supplies

Glass Water Sills: GFL Gesellschaft für Labotechik mbh, Germany.

3.1.5 Kit

Standard Low Molecular Weight Maker Protein: Amersham pharmacia biotech, Sweden.

3.2 Methods

3.2.1 Fungal strains and culture condition

For extracellular protein production, the endophytic fungi were grown in potato dextrose broth purchased from Acumedia Manufacturers Inc., Lansing, MI, USA. For potato dextrose broth preparation, dissolve 24 g of medium in one liter of distilled water. Boil with frequent agitation to completely dissolve the medium and sterilize by autoclaving at 121^0C for 15 minutes. The inoculum was prepared by using 5 pieces of agar culture, which was cut from the growing edge of fungal colonies into 7 mm diameter disks using a flamed cork borer and 250 mL Erlenmeyer flasks containing 100 mL of potato dextrose broth. The inoculum culture was grown for 72 h at room temperature (30-35^0C) and on a rotary shaker (200 rpm). After 72 h, the cultures broth were collected separately by filtration through a No.1 Whatman filter and lyophilized overnight. After lyophilization, the cultures were stored at -20^0C until further analysis.

3.2.2 Extraction of intracellular proteins

The extraction of intracellular proteins from mycelium was prepared from cells frozen under liquid nitrogen, and ground into a fine powder using a mortar and pestle. The powder was dissolved in distilled water. The homogenized mycelia were centrifuged at 13,000×g for 20 min and the supernatant was used as a crude protein for analysis later.

3.2.3 Protein preparation

The total protein concentration of the crude culture samples prior to lyophilization was determined by Bradford assay [16] with bovine serum albumin as a protein standard. After lyophilization, sample were resuspended in 20% trichloroacetic acid (TCA) in acetone (-20^{0}C) and kept at -20^{0}C overnight. After centrifugation at 13,000g at 4^{0}C for 20 minutes, pellets were washed three times with acetone, supernatants were removed by centrifugation and pellets were dried overnight at room temperature**.**

3.2.4 Protein determination

Protein concentration was determined by the Bradford method with bovine serum albumin (BSA) as standard calibration curve. For the quantitative of the protein in this research, micro assay method using a microplate reader has been used. Pipette 10 µl of protein sample into 96 well plates and add 200 µl of the Bradford working solution (Appendix B). Measuring absorbance at 595 nm monitored protein. The concentration of the sample is obtained from a standard curve obtained by using known concentration of standard protein.

3.2.5 Determination of protein molecular weight

1). SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS electrophoresis was performed according to Laemmli. SDS-polyacrylamide gels were cased and run in the compact Hoefer miniVE Vertical Electrophoresis system (8 x 9 cm gels; 1mm thick; 10 wells). Protein molecular weight was measured by SDS-polyacrylamide gel electrophoresis 15%T, 3%C. The SDS-PAGE procedure was performed exactly as described in Chapter 5 of Protein methods text book [16].

(a) Ensure the casting apparatus are clean that consists of a glass plates, two spacers and a comb. Set up the gel cassette; be sure that the bottom of both glass plate and spacers are perfectly flush against a flat surface before tightening clamp assembly.

(b) Prepare the solution (Appendix C) for separating gel. The separating gel is cast first and then overlaid with the stacking gel. Acrylamide is a neurotoxin, so gloves should be worn at all times.

(c) Pipette solution into gel cassette. Carefully the air bubbles becoming trapped within the gel. When the appropriate amount of separating gel solution has been added, about 1-2 cm below the comb is available for the stacking gel.

(d) Overlay the separating gel solution with water to exclude air bubbles. This keeps the gels surface flat. Allow the gel to polymerize for 30-60 min.

(e) When the gel has polymerized, pour off water covering separating gel. Prepare the stacking gel solution (Appendix C) and pipette onto separating gel until solution reaches top of front plate.

(f) Carefully insert comb into the stacking gel, ensuring that no air bubbles are trapped under teeth. Allow the gel to polymerize for at least 30 min.

(g) For sample preparation, four parts of protein samples were mixed with one part of sample buffer (Appendix C) in an eppendorf tube, and heat in boiling water bath for 5 min. Spin down protein solutions for 30 second in microcentrifuge.

(h) After stacking gel has polymerized, remove comb carefully. Load the protein solutions and the molecular weight markers into the well using the gel loading tip. The molecular weight markers were phosphorylase B (97,000 Da), bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), soybean trypsin inhibitor (21,000 Da) and lysozyme (14,400 Da).

(i) Place gel cassette into electrophoresis chamber and pour the electrophoresis buffer (Appendix C) to chamber. Connect the gel cassette to power supply. The lower electrode chamber is connected to the anode and upper one to cathode in SDS-PAGE. The electrophoresis was carried out at 280V and 20 mA for one gel and then was terminated when the dry front reached close to the bottom of the gel.

2). Coomassie Staining

After electrophoresis, proteins in the gel were stained by Coomassie blue. Remove the polyacrylamide gel from between the glass plates and immersed in 50 mL of Coomassie blue staining solution (Appendix C). Place on a rotary shaker and gentle shake the gel 30 minutes. Removed the staining solution and rinse the gel with Coomassie blue destain solution (Appendix C). Place on a rotary shaker 30 minutes and change Coomassie blue destain solution until background of the gel was cleared.

3.2.6 In-gel Digestion

The protein bands were manually excised from the SDS-polyacrylamide gels and transferred to 1.5 ml eppendroff. The gel pieces were washed twice with 200 µl of water for 5 min each. Then, the gel pieces were washed twice with 100 µl of 50%ACN/0.1 M NH_4HCO_3 for 20 min. The liquid was removed, and repeat this step until blue color is washed out. Dried gels by using a speed vacuum centrifuge for 15 min or until gel shrink. Dried gel pieces were swollen in 100 µl of 10 mM DTT /0.1 M NH_4HCO_3 /1 mM EDTA and incubated at 60°C for 45 min to reduce the protein and then the excess DTT solution was removed. The 100 µl of 100 mM iodoacetamide (IAA) /0.1 M NH_4HCO_3 was added and allowed to stand in the dark at room temperature for 30 min. The iodoacetamide solution was removed. The gel pieces were washed twice with 100 µl of 0.05 M Tris-HCl, pH 8.5/50% ACN. Dried gels by using a speed vacuum centrifuge and rehydrate gel in the 180 µl digestion buffer (containing 100 µl of 0.1 M Tris-HCl pH 8.5, 2 µl of 100 mM $CaCl_2$, 20 µl of ACN and 78 µl of distilled water) and 20 µl of trypsin solution and incubated

for overnight at 37°C. After overnight, the trypsin reaction was stopped with adding 20 µl of 2% TFA at 60°C for 30 min. The supernatant was collected and transferred to clean 1.5 ml eppendroff. The peptides were subsequently extracted with 30 µl of digestion buffer to the containing the gel incubated at 30°C for 10 min, sonicated for 5 min. The supernatant was collected and combine in before eppendroff containing supernatant. After that add 30 µl of ACN/digestion buffer (1:1), incubated at 30°C for 10 min and sonicated 5 min. Combine the supernatant in prior eppendroff and add 5% formic acid/ACN in gel 30 µl incubated at 30°C for 10 min, sonicated for 5 min and then collected supernatant. All supernatants were combined and reduced the volume of solution to 20 µl using a speed vacuum centrifuge. These solutions were kept at -20°C for further analysis.

3.2.7 Desalting peptide

Desalting of peptides and proteolytic digests was performed using a 1x15 mm reusable reversed-phase cartridge (green stripe). The first step cleaned cartridge with acetonitrile 500 µl and then the cartridge were equilibrated with 0.1% formic acid in water 300 µl. The peptide sample was loaded on the column and the salts were eluted with 100 to 300 µl of 20% acetonitrile in water, and the peptide was eluted with 200 µl of 0.1% formic acid in 80% acetonitrile in water. Solutions was reduced the volume to 20 µl by using a speed vacuum centrifuge. These solutions were kept at -20°C for further analysis.

For desalting of small volumes of peptide solutions prior to MALDI mass spectrometry C18 ZipTips (Millipore) were employed. Ziptips were moistened with 10 µl of 50% acetonitrile and equilibrated with 0.1% trifluoroacetic acid 10 µl threetimes. The peptides sample was loaded onto the ZipTip, washed with 0.1% trifluoroacetic acid 10 µl three times and eluted with 50% acetonitrile in 0.1% trifluoroacetic acid.

3.2.8 MALDI-TOF mass spectrometry

Mass spectra of protein and peptide were acquired using a MALDI/Tof mass spectrometer (BIFLEX, Bruker, Germany) operating in linear and reflectron modes. The dried samples were dissolved in 50% ACN/ 0.1% TFA and vortexed. The dried droplet method was used for MALDI-MS sample preparation. α-Cyano-4-hydroxycinnamic acid (HCCA) was used as the matrix. For proteins, 1 μl of a sample solution was mixed with 5 μl of a matrix solution (saturated HCCA in 50% ACN/ 0.1% TFA). For peptide mixtures, 1 μl of sample solution was mixed with 1 μl of a matrix solution (saturated HCCA in 50% ACN/ 0.1% TFA). Then, 0.5 μl of the mixture solution was spotted on MALDI target and allowed to dry at room temperature. The MALDI protein mass spectra were performed using a linear mode and calibrated using myoglobin (average mass 16,951 Da) and bovine serum albumin (average mass 66,433.96 Da). MALDI peptide spectra were performed in reflectron mode and externally calibrated using neurotensin (average mass 1,672.9175 Da) and ATCH (average mass 2,465.1983 Da).

CHAPTER IV

RESULTS AND DISCUSSION

Endophytic fungi were identified using all methods as described in the previous chapter. The results were shown and discussed in each part of this chapter, respectively.

4.1 Protein production by endophytic fungi

The endophytic fungi were grown in potato dextrose broth and cultures broths were collected at 1, 3 and 5 days. The extracellular protein production increased rapidly during the first 3 days and remained stationary after 3 days (Figure 4.1). Consequently, the cultures broth were collected at 3 days (72h), it was selected to used for extracellular proteins production in following experiments. For data of protein concentration were presented in Table 1D and 2D (Appendix D).

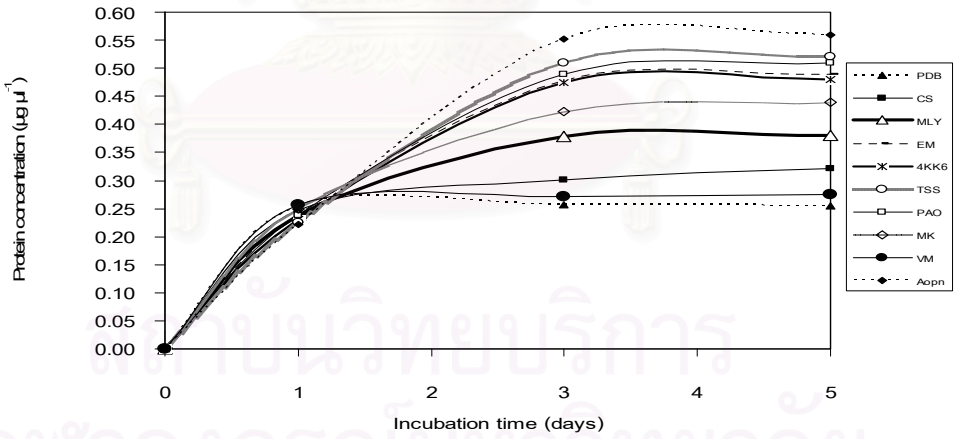


Figure 4.1 Time course of extracellular proteins production by endophytic fungi cultivated in potato dextrose broth (PDB): PDB (control), CS *Bipolaris* sp., MLY *Alternaria* sp., EM *Emericella* sp., 4KK6 Endophytic fungi sp. J48 from coffee leaves., TSS *Nodulispolium* sp., PAO *Glomerrella* sp., MK *Xylaria sp*., VM *Mycoleptodicus* sp. and Aopn Endophytic fungus sp. 00045 5 isolated from oat root. Values are the mean of three replicates.

4.2 Comparative One-dimensional SDS-PAGE of proteins secreted by endophytic fungi

Extracellular proteins produced by endophytic fungi were separated on SDS-PAGE gels using volumes equivalent to 100 mL culture filtrates (Figure 4.2). The SDS-PAGE gels showed the difference of protein patterns in each species. In presence of potato dextrose broth as crude control (lane 1), almost no protein could be detected. While the protein produced from fungal, indicating that protein patterns with a wider range of molecular weights. For example, proteins from *Alternaria* sp. (lane 2) revealed 9 major bands, ranging in molecular weight from 14.4 to 97.0 kDa. Almost of major protein bands of *Alternaria* sp. was estimated between 14.4 to 30.0 kDa and 66.0 to 97.0 kDa, respectively. The protein patterns of *Alternaria* sp. had a specific band when compare with protein patterns of *Bipolaris* sp. (lane 3). The major bands were presented molecular weight between 14.4, 20.1, 30.0 to 45.0 and 66.0 to 97.0 kDa, respectively.

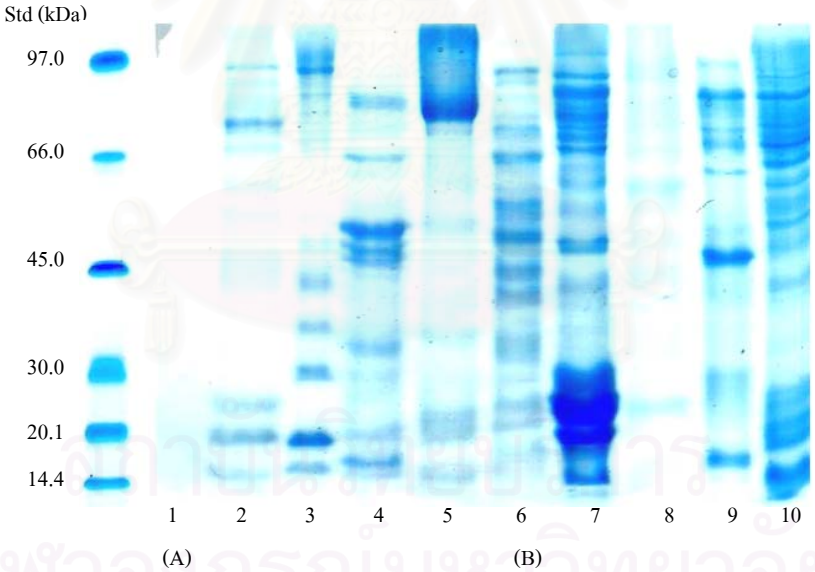


Figure 4.2 One-dimensional SDS-PAGE of crude proteins secreted by endophytic fungal: (A) Potato dextrose broth (control)., (B) Endophytic fungal culture broth; lane 2 *Alternaria* sp., lane 3 *Bipolaris* sp., lane 4 *Mycoleptodicus* sp., lane 5 *Emericella* sp., lane 6 *Xylaria sp*., lane 7 *Nodulispolium* sp., lane 8 Endophytic fungi sp. J48 from coffee leaves lane 9 Endophytic fungus sp. 00045 5 isolated from oat root. lane 10 *Glomerrella* sp.

From the difference of molecular weight of each species can be used for identification of endophytic fungi species. In this research, we studies with the difference of protein molecular weight that indicated by SDS-PAGE gel for three replicate. The results were shown in Figure 1E (Appendix E). Three replicates of SDS-PAGE gels have the similarity of protein patterns.

Intracellular proteins extracted from mycelium were separated on SDS-PAGE gels. The SDS-PAGE gels were shown in Figure 4.3. The SDS-PAGE gels showed the difference of protein patterns in each species. For example, the SDS-PAGE gels of *Bipolaris* sp. showed six individual protein bands. Many bands in SDS-PAGE gels were represented with molecular weight range between 14.4 to 97.0 kDa. From the results, The SDS-PAGE gels of *Alternaria* sp. have more light bands than other species which, indicated that the concentration of protein is lower than limit of detection the technique. From the results were demonstrated above, the SDS-PAGE gels could be able to use for preliminary of protein secretion from endophytic fungi.

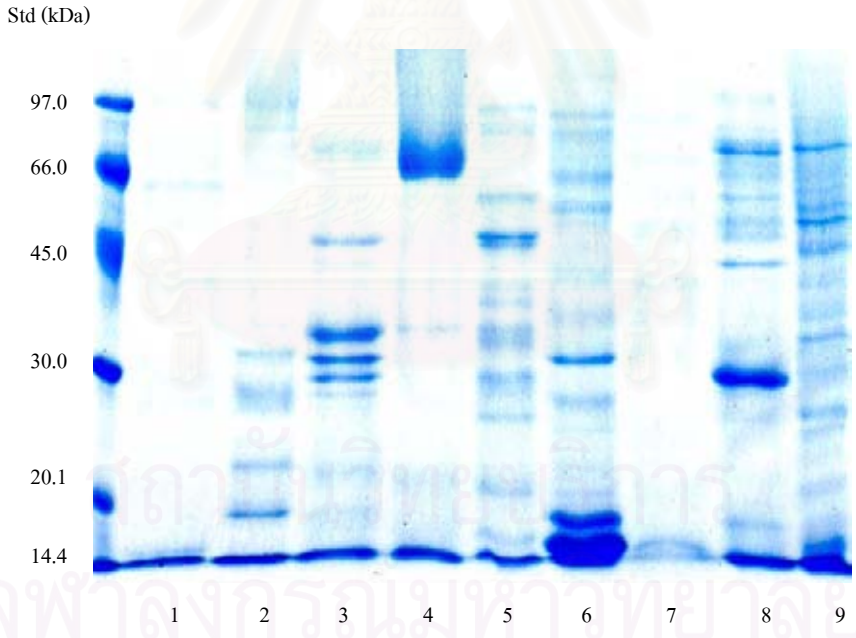


Figure 4.3 One-dimensional SDS-PAGE of crude proteins from mycelium cells: Lane 1 *Alternaria* sp., lane 2 *Bipolaris* sp., lane 3 *Mycoleptodicus* sp., lane 4 *Emericella* sp., lane 5 *Xylaria sp.*, lane 6 *Nodulispolium* sp., lane 7 Endophytic fungi sp. J48 from coffee leaves, lane 8 Endophytic fungus sp. 00045 5 isolated from oat root, lane 9 *Glomerrella* sp.

4.3 Comparison of SDS-PAGE patterns among proteins from crude broth and mycelium cells

From Figure 4.4, The SDS-PAGE gel results indicate that crude proteins from crude broths and mycelium cells had the same major proteins. Protein patterns from crude broths and mycelium cells were showed the similarity of protein patterns. Whereas, protein patterns that specific to each species were also identified.

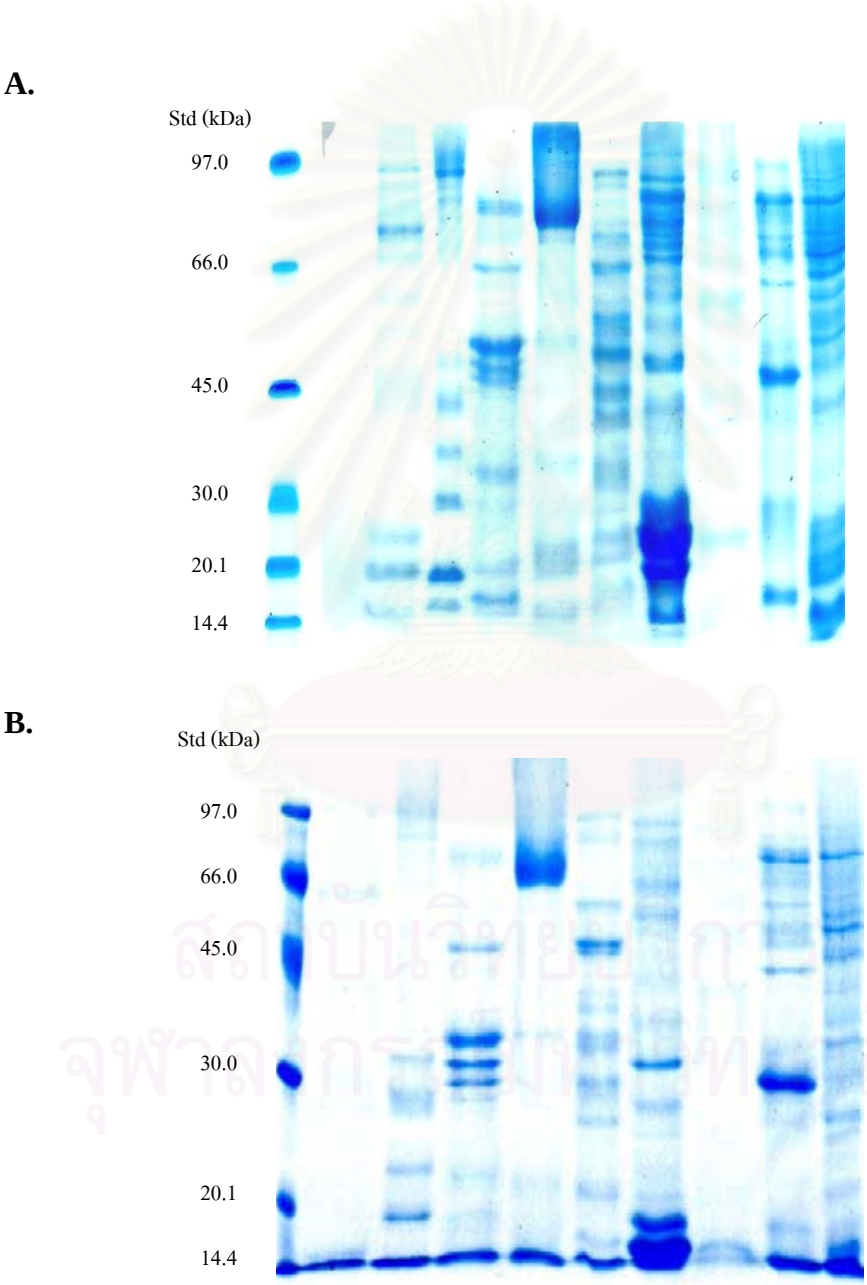


Figure 4.4 One-dimensional SDS-PAGE of proteins secretion from difference extraction sources: A) From crude broth and B) Mycelium cells.

From this result, the SDS-PAGE gels presenting the protein bands that have molecular weight in the vicinity of each species. Therefore, the identification of endophytic fungi was difficult at the strain level. In this study, matrix assisted laser desorption/ionization-time of flight mass spectrometry was used to characterize the protein secreted by endophytic fungi. Proteins from crude broth were selected for characterization due to it has more easily proteins extraction than mycelium cells.

4.4 Matrix Assisted Laser Desorption Ionization-Time of Flight Mass spectrometry analysis

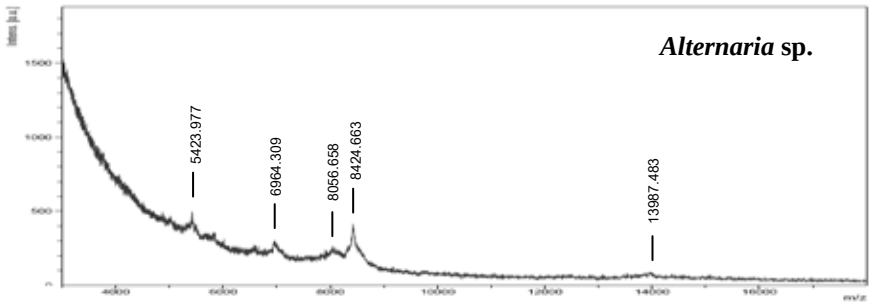
4.4.1 Analysis of crude proteins from endophytic fungi

The proteins from endophytic fungi were analyzed by one-dimensional SDS-PAGE and stained with Coomassie blue. The results shown that the proteins pattern of each endophytic fungi species have specific characteristic. And then, protein samples were analyzed by MALDI-TOF MS. For comparison, the MALDI-TOF spectra for each endophytic fungus between *m/z* of 3,000 and 15,000 are shown in Figure 4.5. The MALDI mass spectrum of *Alternaria* sp. contained the major peaks at *m/z* of 5,423, 6,964, 8056, 8,424 and 13,987, respectively. Table 4.1 is summarizing the *m/z* values observed within the mass spectral of each species of endophytic fungi. The MALDI spectra of endophytic fungi have a specific characteristic when compare with MALDI spectra of potato dextrose broth (control) shown in Figure 1F (Appendix F).

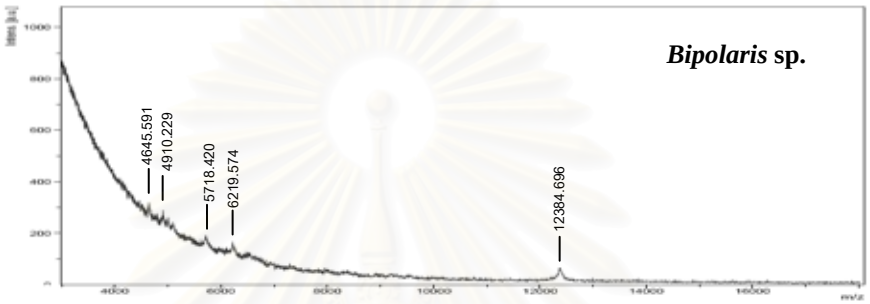
Table 4.1 Summary of mass per charge observed by MALDI-TOF MS from endophytic fungi

species	*m/z*
Alternaria sp.	5423, 6964, 8056, 8424, 13987
Bipolaris sp.	4645, 4910, 5718, 6219, 12384
Xylaria sp.	4184, 8159, 8317
Mycoleptodicus sp.	3559, 4099, 4974, 10659

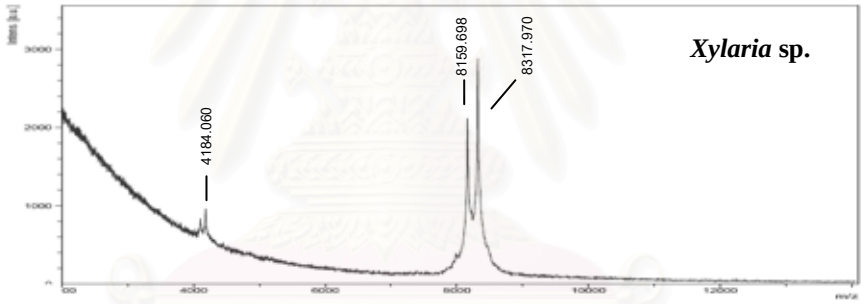
A.



B.



C.



D.

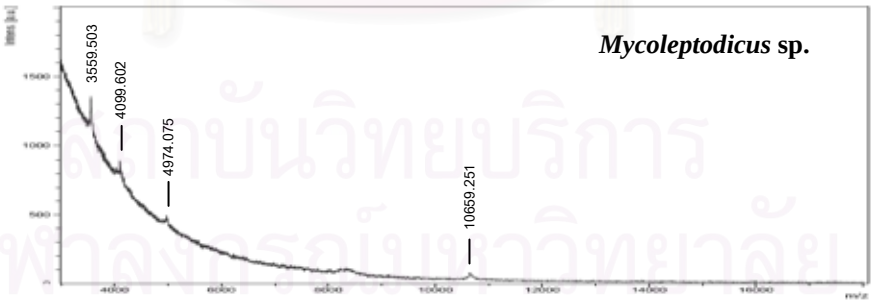


Figure 4.5 MALDI-TOF spectra (*m/z* 3,000 to 18,000) for crude protein from endophytic fungi: A) *Alternaria* sp., B) *Bipolaris* sp., C) *Xylaria* sp. and D) *Mycoleptodicus* sp.

From Figure 4.5, the MALDI spectra results indicate that low intensity peaks. It may be assumed that the concentration of protein is lower than limit of detection the technique. In addition, the majority of proteins secreted from endophytic fungi are highly glycosylated. Glycosylation provides secreted proteins the stability and resistance to environmental influences such as heat, pH effects, and proteolytic attack [50]. Although these protections are necessary for the survival of the proteins, glycosylation poses a problem for their identification by MALDI-TOF MS due to the difficulty of glycoprotein ionization; the tryptic peptide mixture of glycoprotein from each species was used to identify the endophytic fungi.

4.4.2 Analysis of tryptic fragment from endophytic fungi

Molecular weights of tryptic fragments from each species were generated by MALDI-TOF MS in reflectron mode. From the spectra, peak lists were generated using a peak picking algorithm in the Flex Analysis software (Bruker Daltonics). Peak lists were checked to ensure completeness and accuracy, and stored for further analysis. During manual inspection, a mass ion was retained if it met the criteria that the apex had a relative intensity $\geq$ 10%. Mass spectra were representing specific characteristic of molecular weight peptide. The results indicated in Figure 4.6.

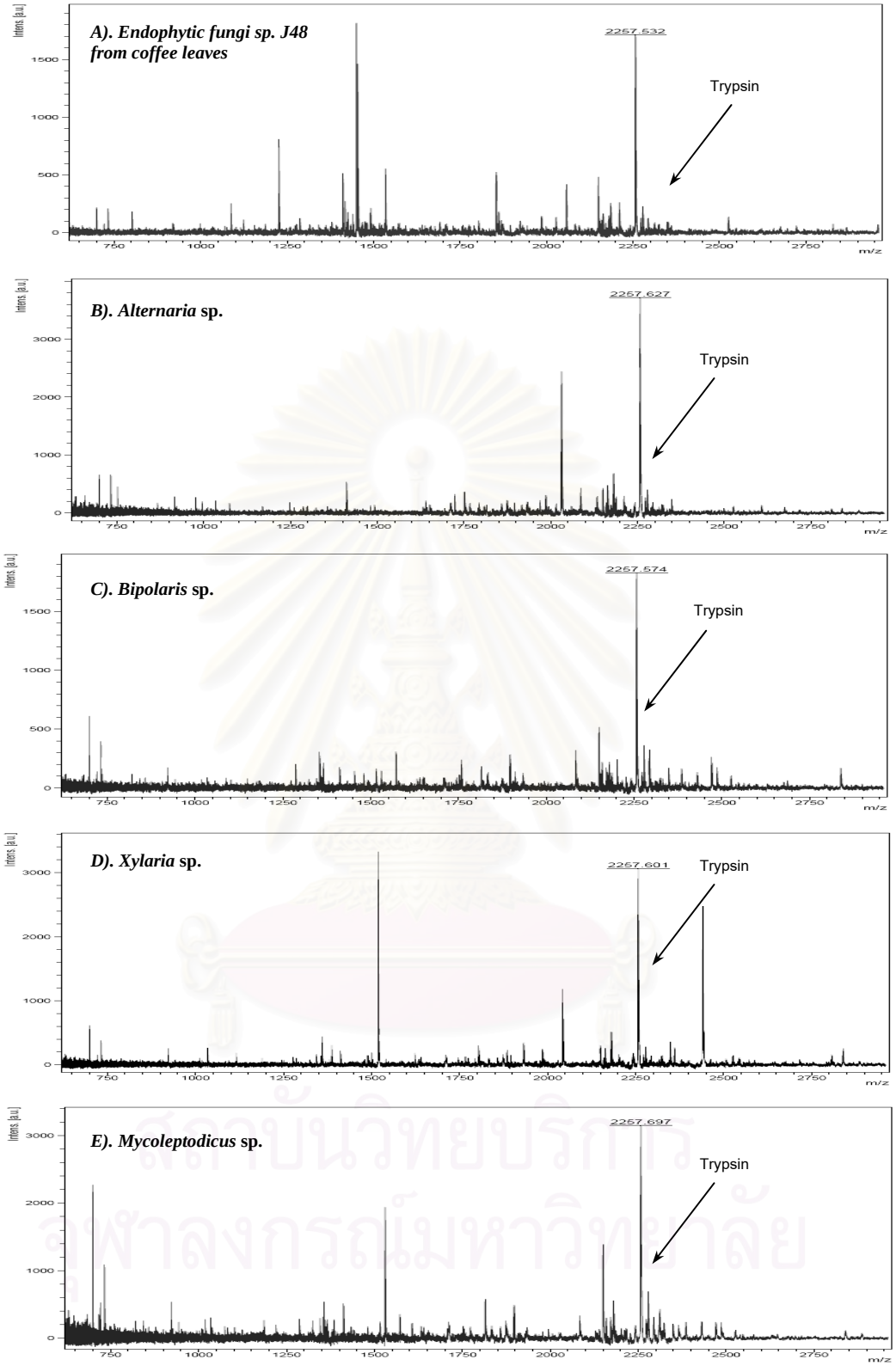


Figure 4.6 MALDI-TOF mass spectra of tryptic fragment of endophytic fungi: A) Endophytic fungi sp. J48 from coffee leaves, B) *Alternaria* sp., C) *Bipolaris* sp., D) *Xylaria* sp. and E) *Mycoleptodicus* sp.

The mass spectra of tryptic fragments are shown in Figure 6F to 10F (Appendix F). Reproducibility of endophyte specie's *m/z* signature was determined by evaluating the frequency of appearance for each ion considered in the mass spectra fingerprint. To insure a reproducible *m/z* signature, ions with at least 10% intensity relative to the largest ion observed in the spectra were considered in the *m/z* signature. The frequency of an ion was calculated by determining the number of times that ion appeared.

The *m/z* signature in mass spectra of each endophytic fungus is shown in Table 4.2 to 4.6. In this research, we study for the tryptic peptide mixture, 3 replicates in each endophytic fungi species. From the results, indicate good repeatability. The calculated values of percent of intensity were collected from each replicate shown the similarity value with small standard deviation. A spectrum was the average of 100 laser shot. The *m/z* signature of the three replicate were compared to determine the reproducibility of each ion, or the frequency of each ion's appeared, as shown in Table 4.2 to 4.6. For example, fifteen ions were considered for *Alternaria* sp., 93% of the ions were 100% reproducible as indicated by a freq = 1.0. Only one ion, *m/z* 2032.139 was 66% reproducible.

Mass spectral were obtained for each of the five species of endophytic fungi used in this study. Table 4.2 to 4.6 is a complication of the *m/z* values and the frequency of the appearance of ions observed within the mass spectral of the each species representing tested. A frequency of 1.00 indicates that the ion is found in all the replicate representing the species.

Representative MALDI spectra for each organism, *Alternaria* sp., Endophytic fungi sp. J48 from coffee leaves, *Bipolaris* sp., *Xylaria* sp. and *Mycoleptodicus* sp., are shown in Table 4.6. The reproducible appearance of *m/z* values is used for our comparison and identification of endophytic fungi approach. The 3 replicate peaks for each species are then compiled, and *m/z* values of each peak are aligned within a standard deviation.

Table 4.2 Reproducibility (*N=3*) of the *m/z* signature of *Alternaria* sp. using MALDI-TOF MS

Replicate 1	Replicate 2	Replicate 3	Stdv	Mean.	Freq[a]	%Intensity[c]
657.513	657.515	657.516	0.002	657.515	1.00	12.781
699.291	699.292	699.292	0.001	699.292	1.00	21.240
732.769	732.771	732.77	0.001	732.770	1.00	23.662
752.513	752.516	752.516	0.002	752.515	1.00	17.209
916.233	916.243	916.233	0.006	916.236	1.00	10.979
977.220	977.219	977.219	0.001	977.219	1.00	11.727
1412.204	1412.204	1412.203	0.001	1412.204	1.00	16.011
1723.537	1723.537	1723.529	0.005	1723.534	1.00	10.450
1750.835	1750.852	1750.863	0.014	1750.850	1.00	11.393
2030.151	2030.179	2030.190	0.020	2030.173	1.00	53.810
2085.523	2085.522	2085.525	0.002	2085.523	1.00	11.124
2163.189	2163.173	2163.248	0.039	2163.203	1.00	10.982
2180.686	2180.688	2180.685	0.002	2180.686	1.00	16.339
2256.646	2256.685	2256.703	0.029	2256.678	1.00	100

[a] Freq = The frequency of appearance of that ion divided by the number of replicate spectra.

[b] N.D. = None detect.

[c] %Intensity = The average of percent of intensity.

Table 4.3 Reproducibility (*N=3*) of the *m/z* signature of Endophytic fungi sp. J48 from coffee leaves using MALDI-TOF MS

Replicate 1	Replicate 2	Replicate 3	Stdv	Mean.	Freq[a]	%Intensity[c]
699.285	699.289	699.292	0.004	699.289	1.00	19.418
732.723	732.745	732.741	0.012	732.736	1.00	16.464
801.936	801.938	801.947	0.006	801.940	1.00	14.414
1088.197	1088.193	1088.212	0.010	1088.201	1.00	19.571
1226.382	1226.403	1226.408	0.014	1226.398	1.00	57.229
1411.333	1411.338	1411.379	0.025	1411.350	1.00	37.251
1418.059	1418.071	1418.084	0.012	1418.071	1.00	18.737
1450.406	N.D.[b]	1450.438	0.023	1450.422	0.66	100
1454.161	1454.178	1454.196	0.018	1454.178	1.00	39.249
1534.308	1534.319	1534.345	0.019	1534.324	1.00	31.868
1854.048	1854.055	1854.091	0.023	1854.065	1.00	23.641
2057.217	2057.238	2057.268	0.026	2057.241	1.00	79.821
2256.519	2256.549	2256.582	0.032	2256.550	1.00	19.418
N.D.	2257.544	2257.538	0.004	2257.541	0.66	16.464

[a] Freq = The frequency of appearance of that ion divided by the number of replicate spectra.

[b] N.D. = None detect.

[c] %Intensity = The average of percent of intensity.

Table 4.4 Reproducibility (*N=3*) of the *m/z* signature of *Bipolaris* sp. using MALDI-TOF MS

Replicate 1	Replicate 2	Replicate 3	Stdv	Mean.	Freq[a]	%Intensity[c]
630.655	630.686	630.687	0.018	630.676	1.00	19.676
699.244	699.251	699.251	0.004	699.248	1.00	100
732.734	732.738	732.739	0.003	732.737	1.00	60.638
736.726	736.718	736.717	0.005	736.720	1.00	17.912
820.565	820.561	820.562	0.002	820.563	1.00	17.547
922.283	922.276	922.275	0.004	922.278	1.00	27.882
1287.121	1287.120	1287.120	0.001	1287.120	1.00	31.475
1354.594	1354.611	1354.615	0.011	1354.607	1.00	39.275
1356.423	1356.443	1356.443	0.012	1356.436	1.00	17.606
1412.136	1412.130	1412.131	0.003	1412.132	1.00	27.417
1454.288	1454.301	1454.304	0.009	1454.298	1.00	21.356
1480.588	1480.578	1480.577	0.006	1480.581	1.00	18.622
1515.804	1515.828	1515.844	0.020	1515.825	1.00	25.759
1530.333	1530.339	1530.337	0.003	1530.336	1.00	22.297
1572.299	1572.297	1572.295	0.002	1572.297	1.00	44.698
1758.585	1759.532	1759.517	0.542	1759.211	1.00	24.084
1815.061	1815.068	1815.071	0.005	1815.067	1.00	25.050
1896.814	1896.803	1896.815	0.007	1896.811	1.00	26.977
2158.352	2158.366	2158.364	0.008	2158.361	1.00	15.251
2178.72	2178.688	2178.692	0.017	2178.700	1.00	15.544
2201.136	2201.179	2201.187	0.027	2201.167	1.00	21.568
2277.877	2277.913	2277.945	0.034	2277.912	1.00	32.106
2293.393	2293.429	2293.440	0.025	2293.421	1.00	29.515
2470.058	2470.101	2470.187	0.066	2470.115	1.00	22.500

[a] Freq = The frequency of appearance of that ion divided by the number of replicate spectra.

[b] N.D. = None detect.

[c] %Intensity = The average of percent of intensity.

Table 4.5 Reproducibility (*N=3*) of the *m/z* signature of *Xylaria* sp. using MALDI-TOF MS

Replicate 1	Replicate 2	Replicate 3	Stdv	Mean.	Freq[a]	%Intensity[c]
699.283	699.269	699.283	0.008	699.278	1.00	20.723
732.748	732.736	732.751	0.008	732.745	1.00	12.190
1359.494	1359.478	1359.491	0.008	1359.488	1.00	13.433
1518.725	1518.706	1518.727	0.012	1518.719	1.00	100
1386.842	1386.833	1386.844	0.006	1386.840	1.00	9.076
2042.741	2042.729	2042.766	0.019	2042.745	1.00	27.631
2180.641	2180.617	2180.617	0.014	2180.625	1.00	10.573
2256.652	2256.596	2256.71	0.057	2256.653	1.00	68.466
2439.989	2439.969	2440.076	0.057	2440.011	1.00	50.841

[a] Freq = The frequency of appearance of that ion divided by the number of replicate spectra.

[b] N.D. = None detect.

[c] %Intensity = The average of percent of intensity.

Table 4.6 Reproducibility (*N=3*) of the *m/z* signature of *Mycoleptodicus* sp. using MALDI-TOF MS

Replicate 1	Replicate 2	Replicate 3	Stdv	Mean.	Freq[a]	%Intensity[c]
624.768	624.774	624.774	0.003	624.772	1.00	13.616
699.274	699.278	699.278	0.002	699.277	1.00	100
716.866	716.871	716.873	0.004	716.870	1.00	13.836
720.980	720.976	720.976	0.002	720.977	1.00	21.197
922.291	922.297	922.293	0.003	922.294	1.00	22.559
1019.419	1019.409	1019.414	0.005	1019.414	1.00	11.098
1035.132	1035.135	1035.131	0.002	1035.133	1.00	11.913
1186.228	1186.221	1186.221	0.004	1186.223	1.00	9.833
1220.489	1220.485	1220.490	0.003	1220.488	1.00	8.851
1287.120	1287.116	1287.115	0.003	1287.117	1.00	11.512
1345.694	1345.704	1345.700	0.005	1345.699	1.00	10.894
1354.628	1354.617	1354.620	0.006	1354.622	1.00	12.726
1356.456	1356.444	1356.449	0.006	1356.450	1.00	18.025
1384.878	1384.876	1384.874	0.002	1384.876	1.00	10.162
1412.162	1412.145	1412.146	0.010	1412.151	1.00	22.409
1515.777	1515.806	1515.801	0.016	1515.795	1.00	9.823
1530.353	1530.360	1530.356	0.004	1530.356	1.00	82.559
1572.327	1572.315	1572.312	0.008	1572.318	1.00	14.244
1815.060	1815.072	1815.070	0.006	1815.067	1.00	23.747
1894.824	N.D.[b]	1894.809	0.011	1894.817	0.66	8.683
2178.763	2178.967	2178.910	0.105	2178.880	1.00	16.307
2256.797	2256.952	2256.963	0.093	2256.904	1.00	93.193
2277.932	2278.463	2278.534	0.329	2278.310	1.00	12.804

[a] Freq = The frequency of appearance of that ion divided by the number of replicate spectra.

[b] N.D. = None detect.

[c] %Intensity = The average of percent of intensity.

CHAPTER V

CONCLUSION

In this research, the identification of endophytic fungi was carried out by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS). Nine species of endophytic fungi used in this study was obtained from the Research Centre for Bioorganic Chemistry (RCBC, Chulalongkorn University, Thailand). The fungi secreted extracellular proteins rapidly during the first 3 days but remained stationary after 3 days. Thus, the fungi were collected to be used for extracellular proteins analysis in 3 days.

Crude proteins from broth and mycelium were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis. The SDS-PAGE results of each species were indicated individual separate protein bands. The comparison of SDS-PAGE patterns between proteins from crude broth and fungal mycelia were showed to have similarity of proteins pattern that it could be to use for preliminary identified of endophytic fungi. The crude proteins were analyzed by using MALDI-TOF MS. The results revealed different characteristic of mass spectrum of each species. However, the MALDI spectra results indicated low intensity peaks. It may be assumed that the concentration of protein was lower than limit of detection the technique. In addition, the majority of proteins secreted from endophytic fungi were highly glycosylated. Thus, in this research we studies with the molecular weight of peptide mixture from each species was generated by MALDI-TOF MS.

From the result of mass spectra of tryptic fragments, each species of endophytic fungi represented gave difference mass spectrum. The reproducible appearance of *m/z* values is used for comparison and identification of endophytic fungi approach.

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APPENDICES

Appendix A

Media

A. Potato dextrose broth (PDB)

Purchased from Acumedia Manufacturers Inc., Lansing, MI, USA

Formula/Liter

Potato Infusion Solids	4 g
Dextrose	20 g

Final pH: 5.1 ± 0.2 at 25 ^{0}C

Directions

1. Dissolve 24 g of medium in one liter of distilled water.
2. Heat with frequent agitation to completely dissolve the medium.
3. Autoclaving at 121^{0}C for 15 minutes.

B. Potato dextrose agar (PDA)

Directions

1. Dissolve 24 g of medium in one liter of distilled water.
2. Heat with frequent agitation to completely dissolve the medium.
3. Add 15 g of agar and dissolve by steaming.
4. Autoclaving at 121^{0}C for 15 minutes.

Appendix B

Solutions in Bradford Assay

A rapid and reliable dye based assay for determining protein content in a solution. This assay is sometimes referred to as the Bio-Rad assay after the company which sells a widely used kit that is based on this method.

A. Bradford stock solution

	Final concentration	**Amount**
Ethanol	95% (v/v)	100 ml
Phosphoric acid	88% (v/v)	200 ml
Serva Blue G		350 mg

B. Bradford working buffer

	Final concentration	**Amount**
Ethanol	95% (v/v)	15 ml
Phosphoric acid	88% (v/v)	30 ml
Bradford stock solution		30 ml
Distilled water		to 500 ml

Filter through Whatman No. 1 paper, store at room temperature in brown glass bottle. Usable for several weeks, but may need to be refiltered.

Appendix C

Solutions in SDS-PAGE

The acrylamide, N, N'- methylenebisacrylamide, TEMED, ammonium persulfate and SDS in this experiments are extremely hazardous. Hazardous materials should be weighed in a fume hood while wearing a disposable dust mask and using double latex gloves for all protocols.

I. Stock Solutions

A. 2 M Tris-HCl (pH 8.8), 100 ml

	Final concentration	**Amount**
Tris-base	2 M	24.2 g
Distilled water		50 ml
HCl		adjust to pH 8.8
Distilled water		to 100 ml

Allow solution to cool to room temperature, pH will increase.

B. 1 M Tris-HCl (pH 6.8), 100 mL

	Final concentration	**Amount**
Tris-base	1 M	12.1 g
Distilled water		50 ml
HCl		adjust to pH 6.8
Distilled water		to 100 ml

Allow solution to cool to room temperature, pH will increase.

C. 10% SDS (w/v), 100 mL

	Final concentration	**Amount**
SDS	10% (w/v)	10 g
Distilled water		to 100 ml

Store at room temperature.

D. 50% glycerol (v/v), 100 ml

	Final concentration	Amount
100% glycerol	50% (v/v)	50 ml
Distilled water		to 50 ml

E. 1% bromophenol blue (w/v), 10 ml

	Final concentration	Amount
Bromophenol blue	1% (w/v)	100 mg
Distilled water		to 10 ml

Filtration will remove aggregated dye.

II. Working Solutions

A. Solution A (acrylamide stock solution)

(30% acrylamide, 0.8% N, N'- methylenebisacrylamide)

	Final concentration	Amount
Acrylamide	30% (w/v)	29.2 g
N, N'- methylenebisacrylamide	0.8% (w/v)	0.8 g
Distilled water		to 100 ml

Solution can be stored for months in the refrigerator.

B. Solution B (4x separating gel buffer)

(2 M Tris-HCl, pH 8.8, 10% SDS)

	Final concentration	Amount
2 M Tris-HCl (see stock solution A)	1.5 M	75 ml
10% SDS (see stock solution C)	0.4% (v/v)	4 ml
Distilled water		to 100 ml

Solution can be stored for months in the refrigerator.

C. Solution C (4x stacking gel buffer)

(1 M Tris-HCl, pH 6.8, 10% SDS)

	Final concentration	**Amount**
1 M Tris-HCl (see stock solution B)	0.5 M	50 ml
10% SDS (see stock solution C)	0.4% (v/v)	4 ml
Distilled water		to 100 ml

Solution can be stored for months in the refrigerator.

D. 10% Ammonium persulfate

	Final concentration	**Amount**
Ammonium persulfate	10%	0.5 g
Distilled water		to 5 ml

Stable for months in a capped tube in the refrigerator.

E. Electrophoresis buffer

	Final concentration	**Amount**
Tris-base	25 mM	3 g
Glycine	192 mM	14.4 g
SDS	0.1% (w/v)	1 g
Distilled water		to 1000 ml

pH should be approximately 8.3

F. 5x Sample buffer

(*1 M Tris-HCl, pH 6.8, 50% glycerol, 10% SDS, 2-mercaptoethanol)*

	Final concentration	**Amount**
1 M Tris-HCl (see stock solution B)	60 mM	0.6 ml
50% glycerol (see stock solution D)	25% (v/v)	5 ml
10% SDS (see stock solution C)	2% (v/v)	2 ml
2-mercaptoethanol	14.4 mM	0.5 ml
Distilled water		to 10 ml

Stable for weeks in the refrigerator or for mouths at -20^0 C.

III. Coomassie gel stain

	Amount
Coomassie blue R-250	1.0 g
Methanol	450 ml
Glacial acetic acid	100 ml
Distilled water	450 ml

IV. Coomassie gel destain

	Amount
Methanol	100 ml
Glacial acetic acid	100 ml
Distilled water	800 ml

V. Gel preparation

	Separating gel (10 ml)	Stacking gel (4 ml)
Solution A	5.0 ml	0.67 ml
Solution B	2.5 ml	-
Solution C	-	1.0 ml
10% Ammonium persulfate	50 µl	30 µl
TEMED	5.0 µl	5.0 µl
Distilled water	2.5 ml	2.3 ml

10% Ammonium persulfate and TEMED are added in last step because polymerization will be under way.

Appendix D

Table 1D The total protein concentration of endophytic fungi from crude broth.

Species	Protein concentration (µg/µl)[a]		
	Day 1	Day 3	Day 5
PDB (control)	0.254	0.256	0.255
Mycoleptodicus sp.	0.256	0.271	0.275
Bipolaris sp.	0.248	0.301	0.322
Alternaria sp.	0.239	0.378	0.380
Xylaria sp.	0.250	0.424	0.440
Endophytic fungi sp. J48 from coffee leaves	0.230	0.474	0.440
Emericella sp.	0.240	0.478	0.490
Glomerrella sp.	0.238	0.490	0.510
Nodulispolium sp.	0.225	0.509	0.520
Endophytic fungus sp. 00045 5 isolated from oat root	0.221	0.552	0.559

[a] *Protein concentration were determined 3 replicate for each days.*

Table 2D The total protein concentration of endophytic fungi from mycelium cells.

Species	Protein concentration (µg/µl)[a]		
	Day 1	Day 3	Day 5
Mycoleptodicus sp.	0.267	0.275	0.283
Bipolaris sp.	0.309	0.318	0.321
Alternaria sp.	0.378	0.383	0.382
Xylaria sp.	0.449	0.458	0.462
Endophytic fungi sp. J48 from coffee leaves	0.476	0.483	0.485
Emericella sp.	0.468	0.479	0.490
Glomerrella sp.	0.480	0.499	0.489
Nodulispolium sp.	0.515	0.520	0.528
Endophytic fungus sp. 00045 5 isolated from oat root	0.571	0.568	0.570

[a] *Protein concentration were determined 3 replicate for each days.*

Appendix E

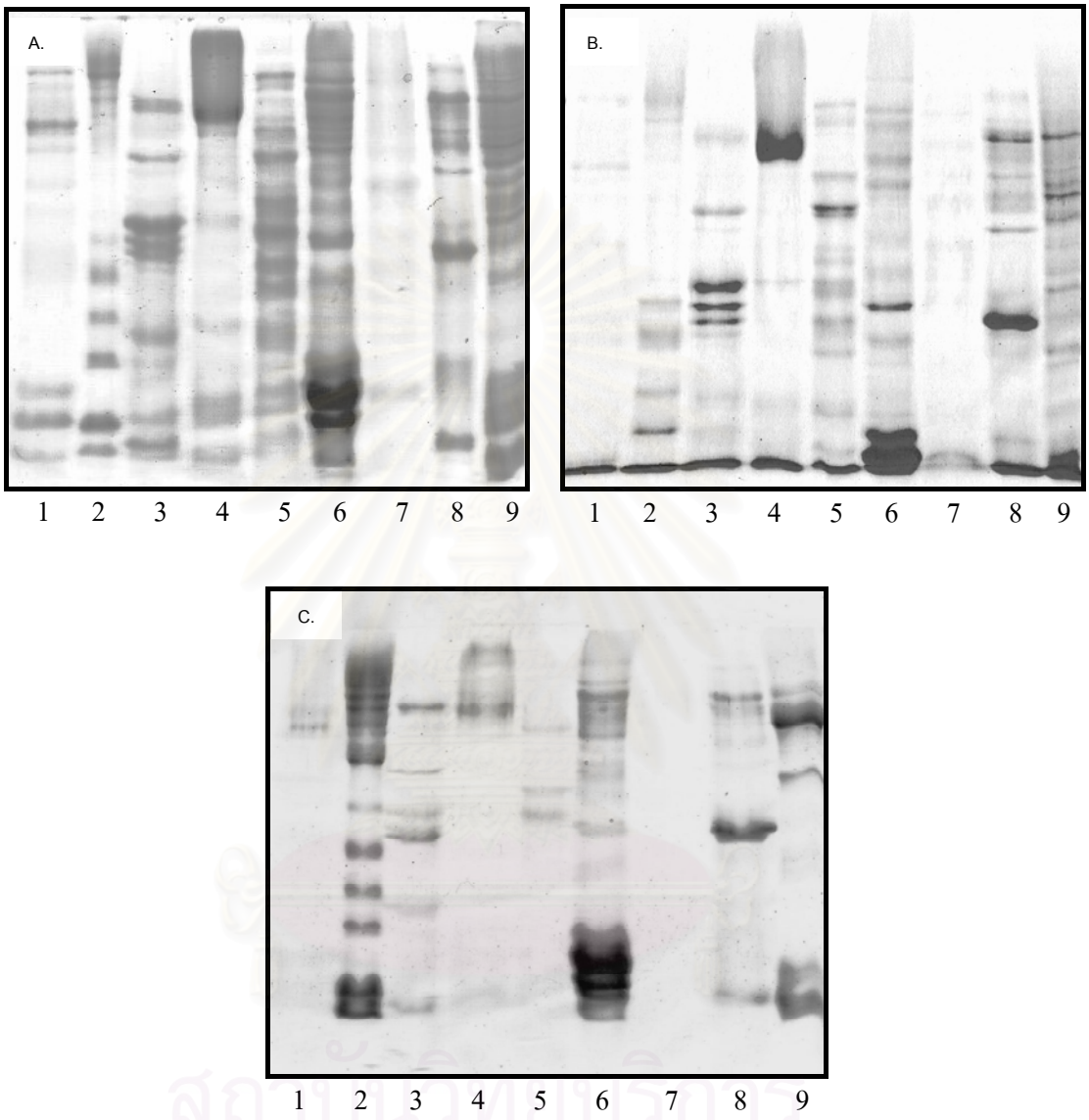


Figure 1E One-dimensional SDS-PAGE of crude proteins secreted by endophytic fungal: (A) Replicate 1, (B) Replicate 2, (C) Replicate 3; Lane 1 *Alternaria* sp., lane 2 *Bipolaris* sp., lane 3 *Mycoleptodicus* sp., lane 4 *Emericella* sp., lane 5 *Xylaria sp.*, lane 6 *Nodulispolium* sp., lane 7 Endophytic fungi sp. J48 from coffee leaves, lane 8 Endophytic fungus sp. 00045 5 isolated from oat root, lane 9 *Glomerrella* sp.

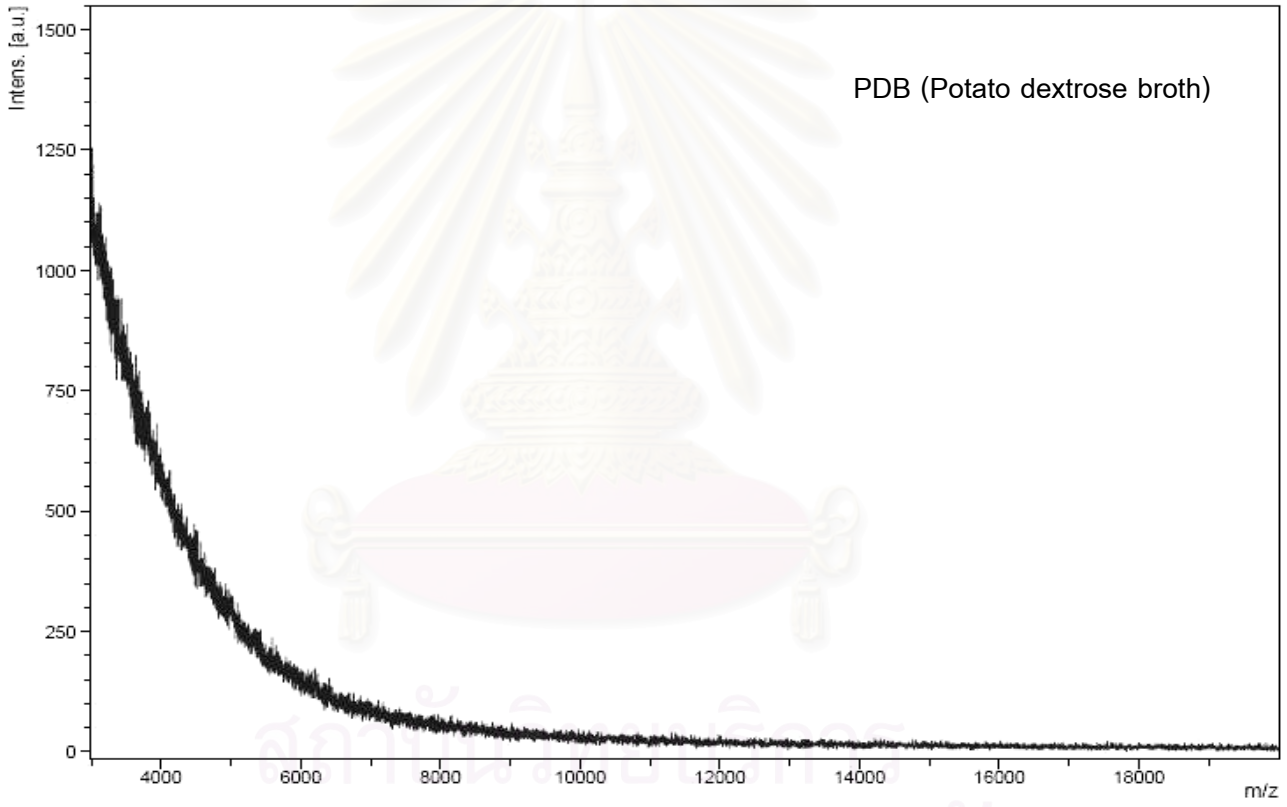


Figure 1F MALDI-TOF MS spectrum (*m/z* 4,000 to 18,000) for Potato dextrose broth

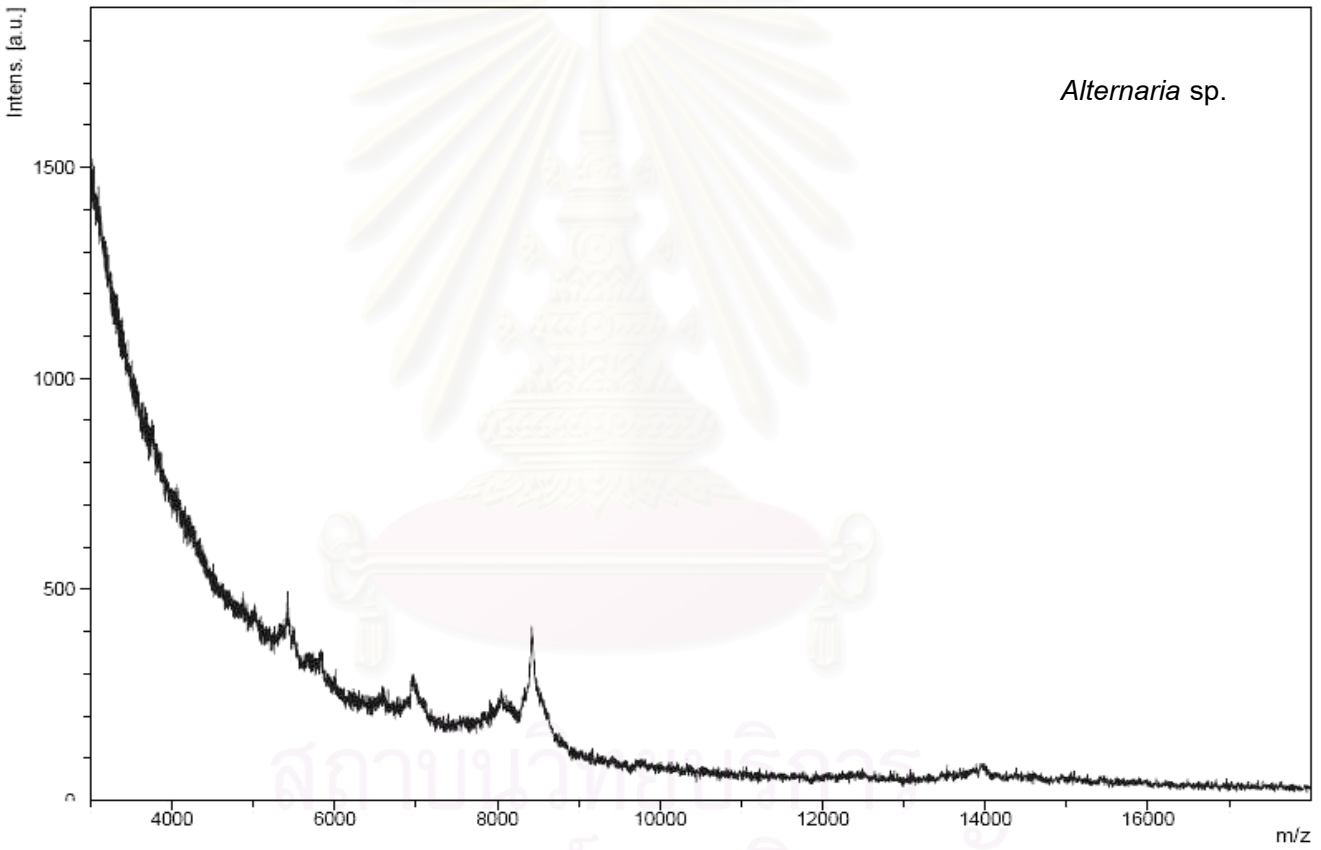


Figure 2F MALDI-TOF MS spectrum (*m/z* 4,000 to 18,000) for *Alternaria* sp.

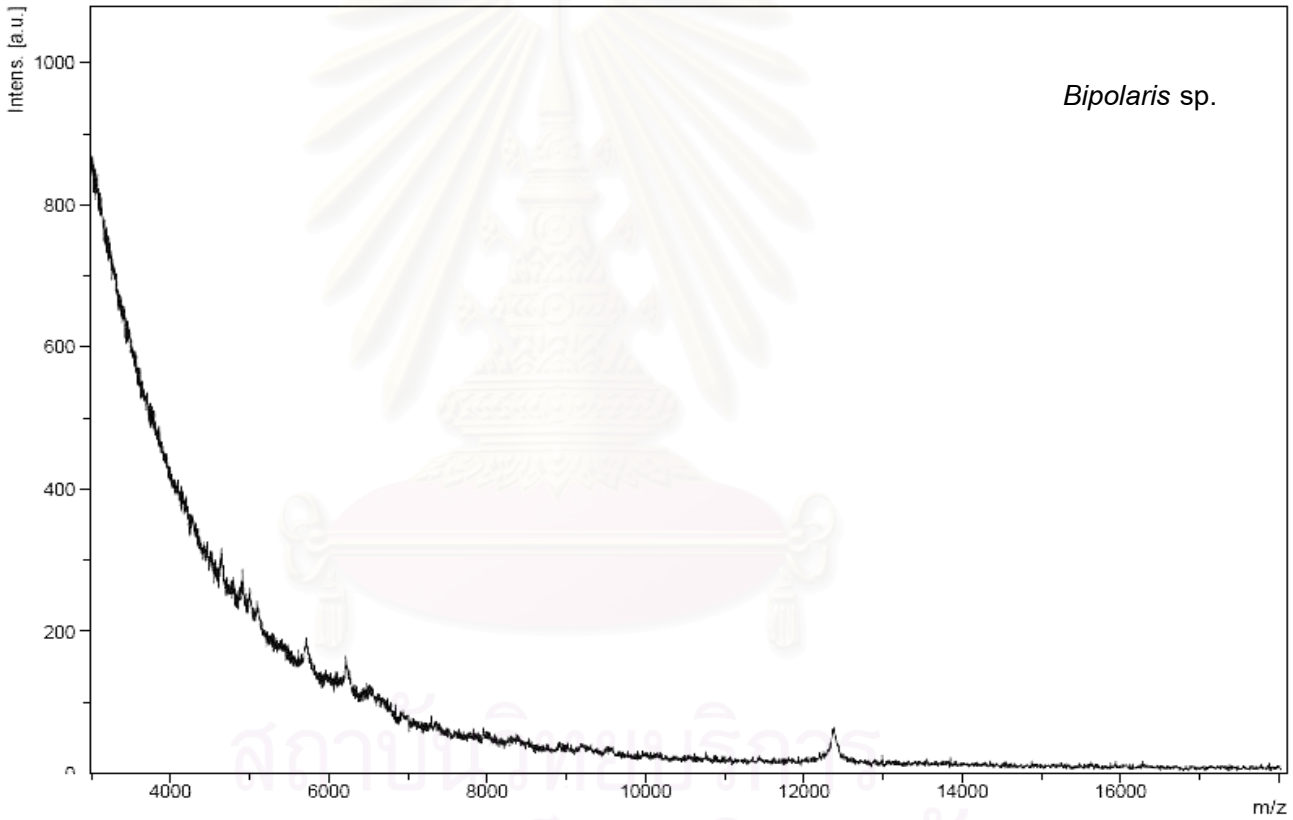


Figure 3F MALDI-TOF MS spectrum (*m/z* 4,000 to 18,000) for *Bipolaris* sp.

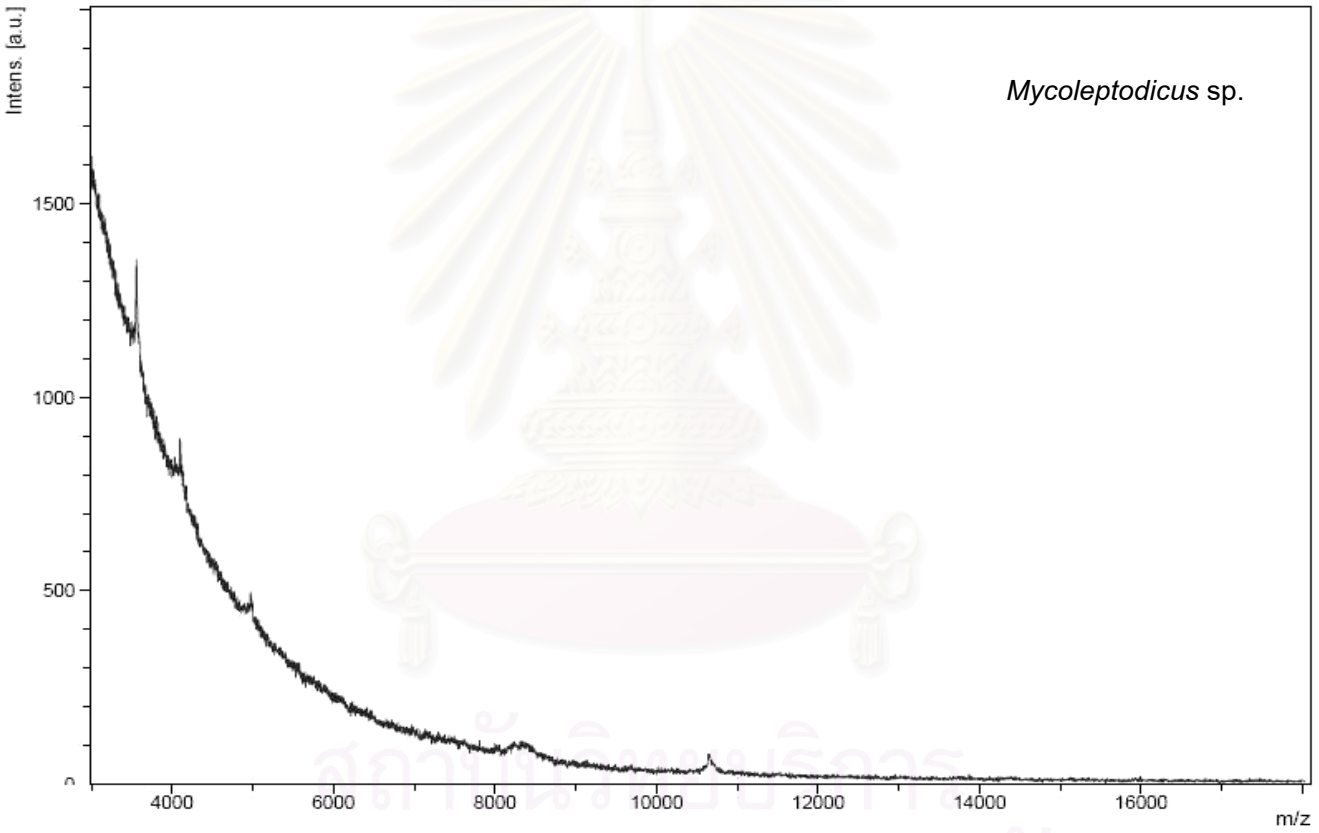


Figure 4F MALDI-TOF MS spectrum (*m/z* 4,000 to 18,000) for *Mycoleptodicus* sp.

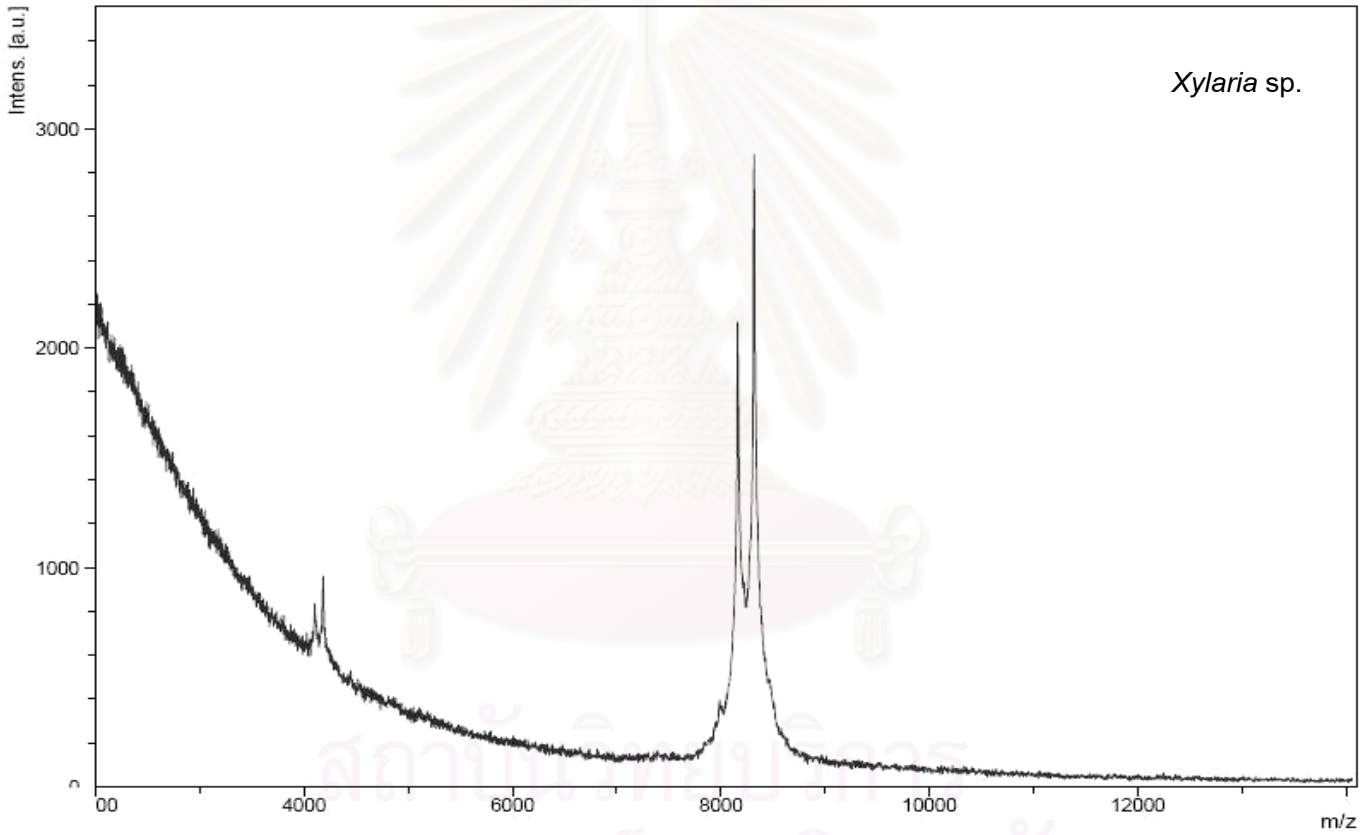
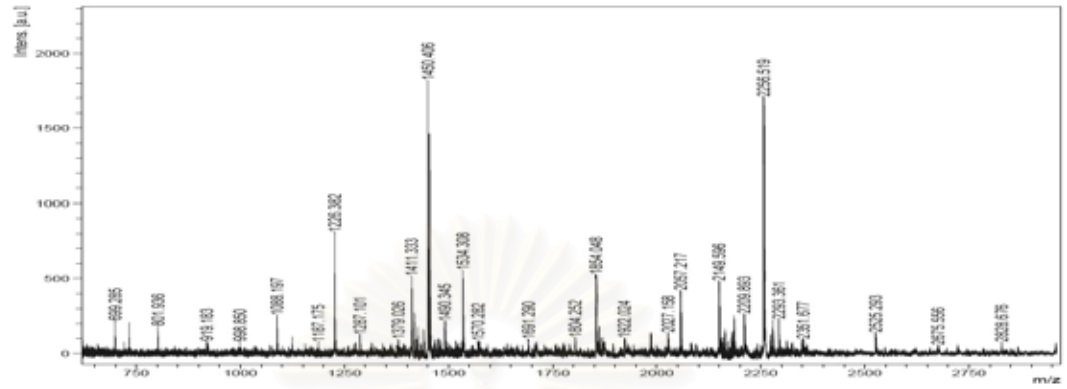


Figure 5F MALDI-TOF MS spectrum (*m/z* 4,000 to 18,000) for *Xylaria* sp.

A.



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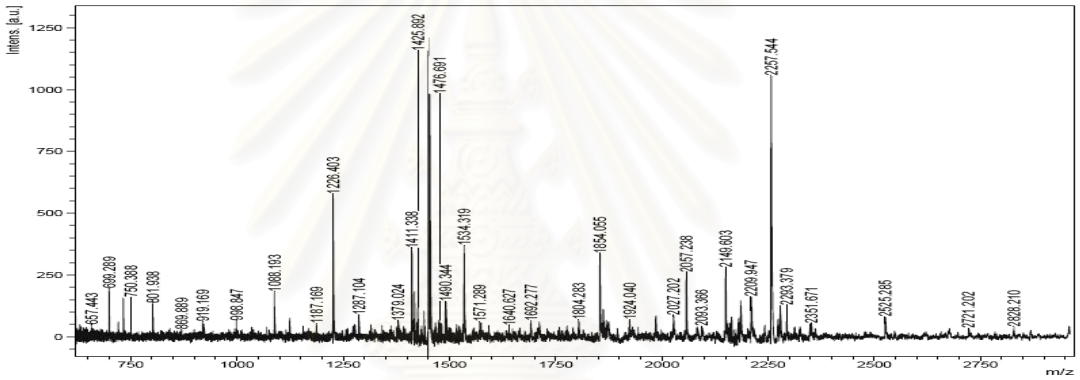


C.

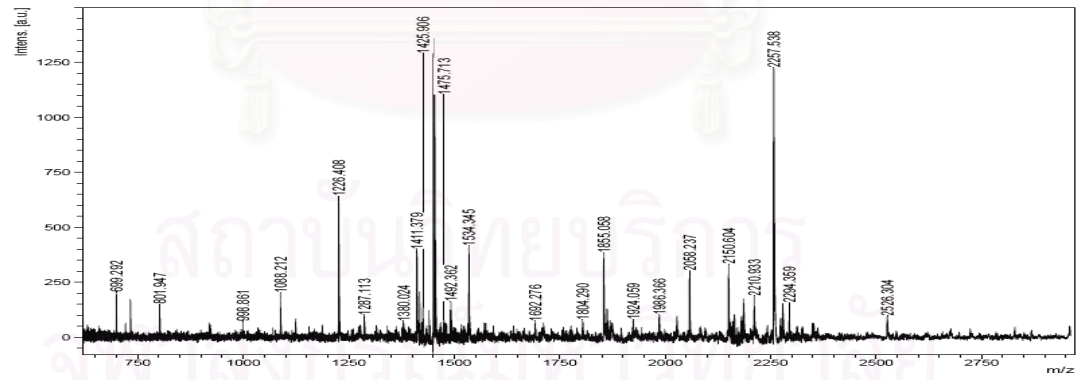
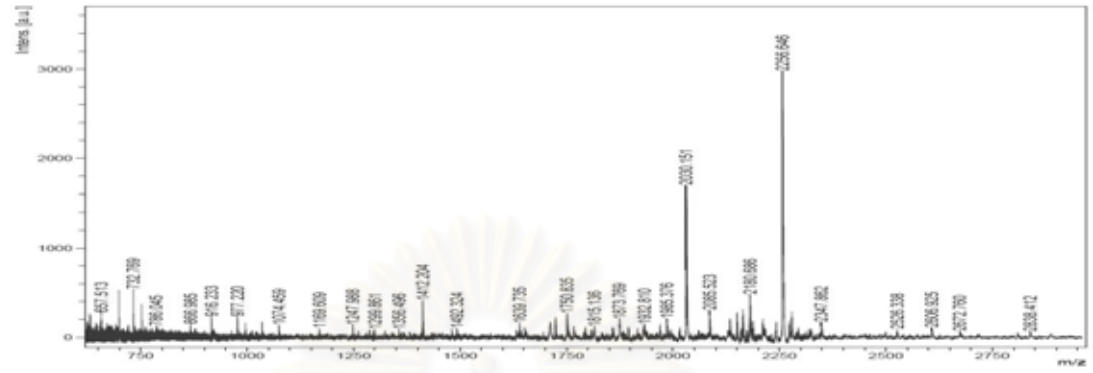


Figure 6F MALDI-TOF mass spectra of tryptic fragment of endophytic fungi sp. J48 from coffee leaves: A) Replicate 1, B) Replicate 2 and C) Replicate 3.

A.



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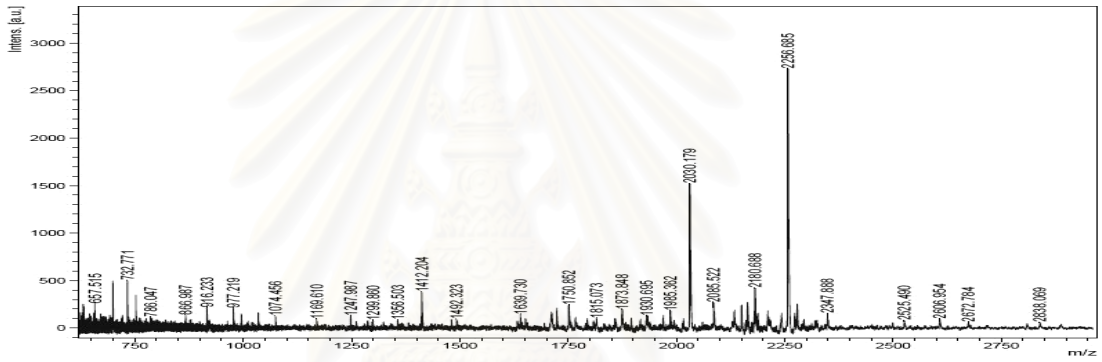

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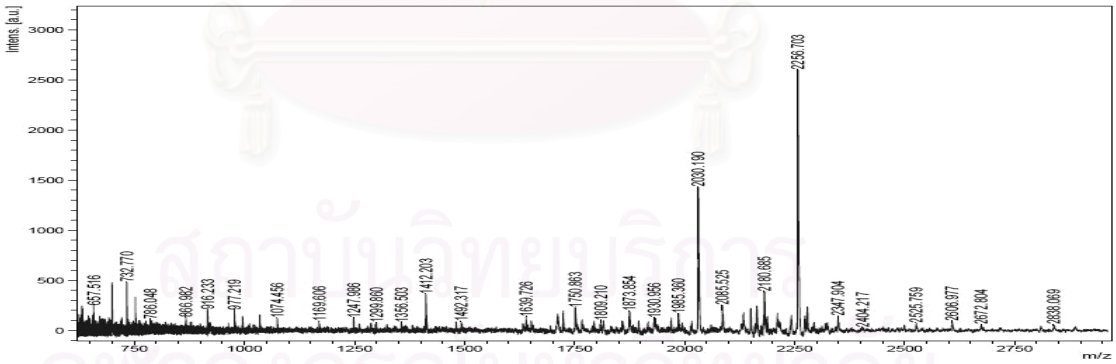
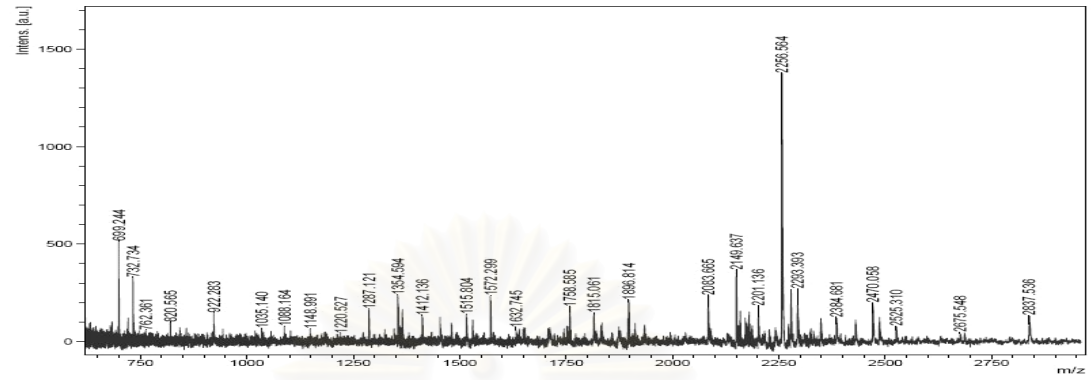


Figure 7F MALDI-TOF mass spectra of tryptic fragment of *Alternaria* sp.: A) Replicate 1, B) Replicate 2 and C) Replicate 3.

A.



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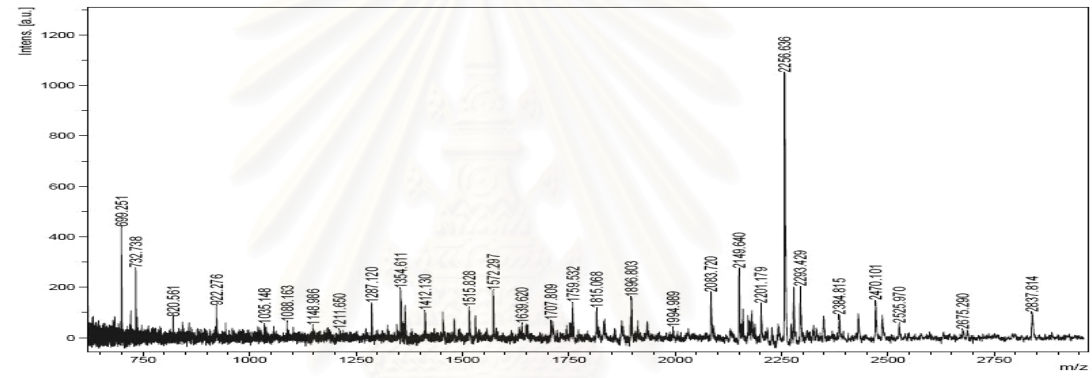


C.

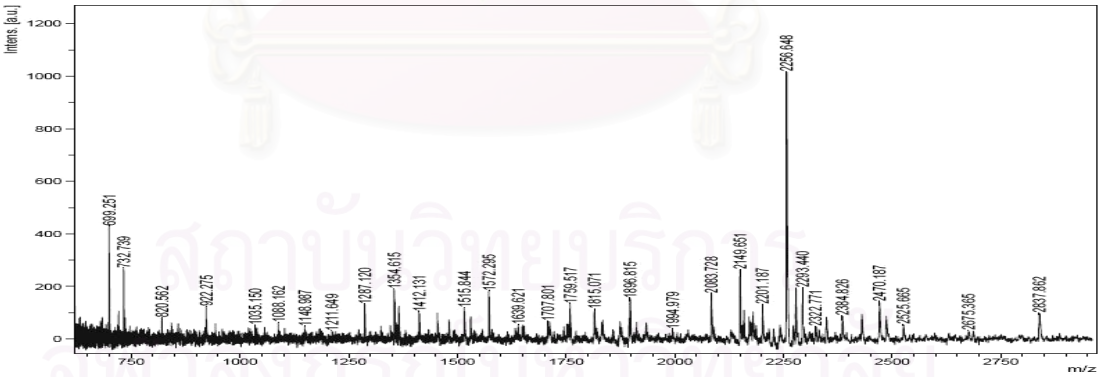
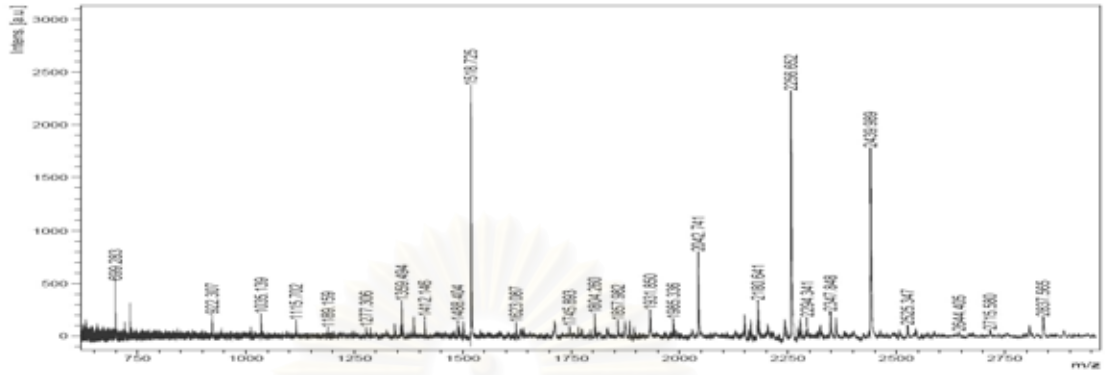


Figure 8F MALDI-TOF mass spectra of tryptic fragment of *Bipolaris* sp.: A) Replicate 1, B) Replicate 2 and C) Replicate 3.

A.



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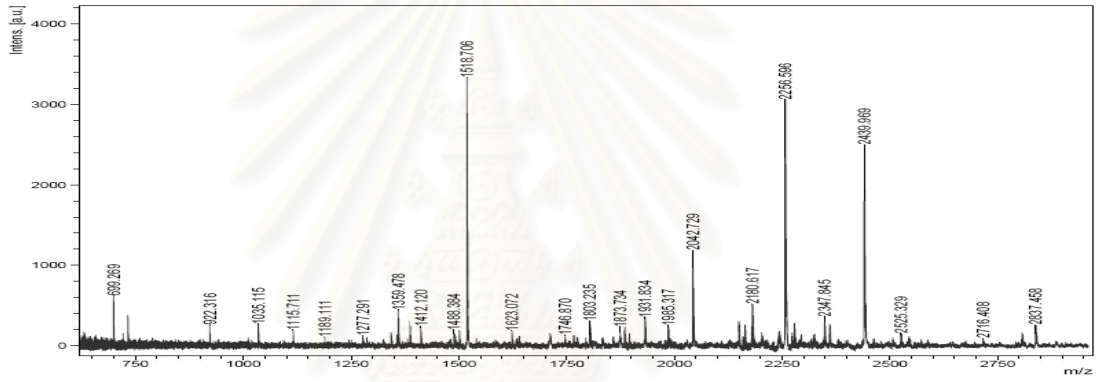

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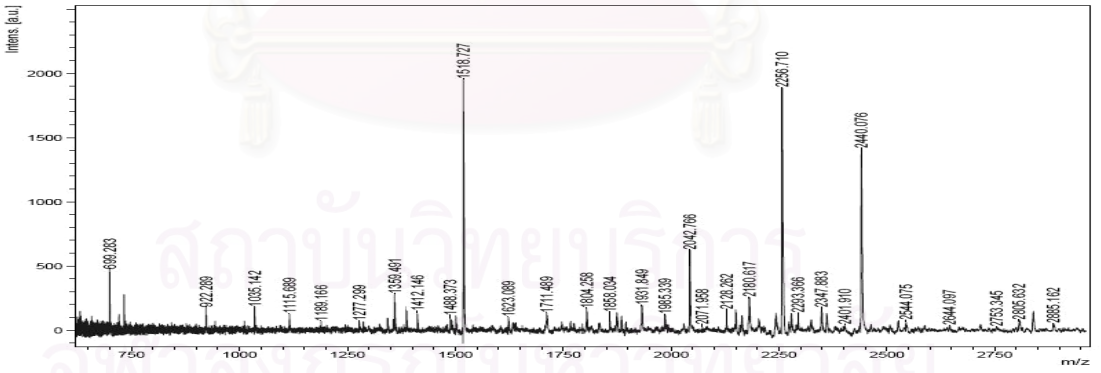
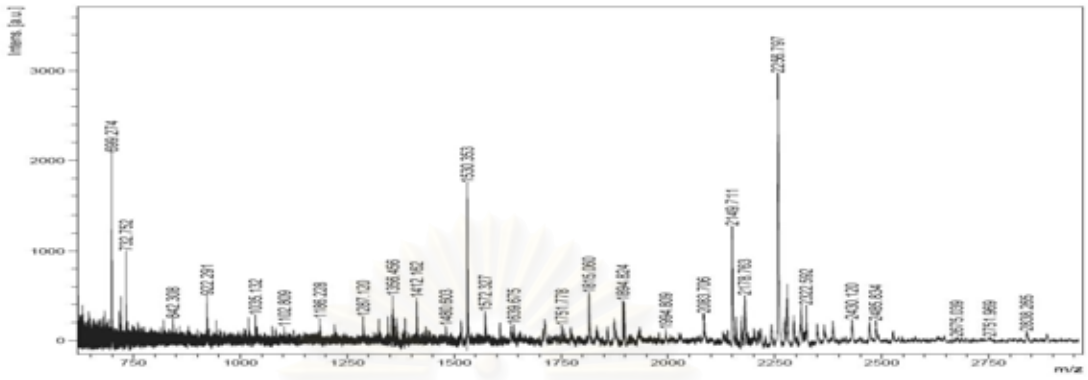
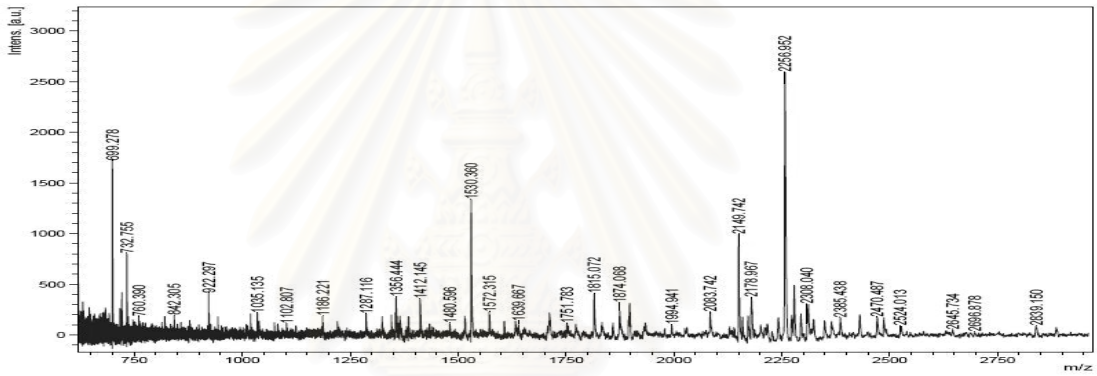


Figure 9F MALDI-TOF mass spectra of tryptic fragment of *Xylaria* sp.: A) Replicate 1, B) Replicate 2 and C) Replicate 3.

A.



B.



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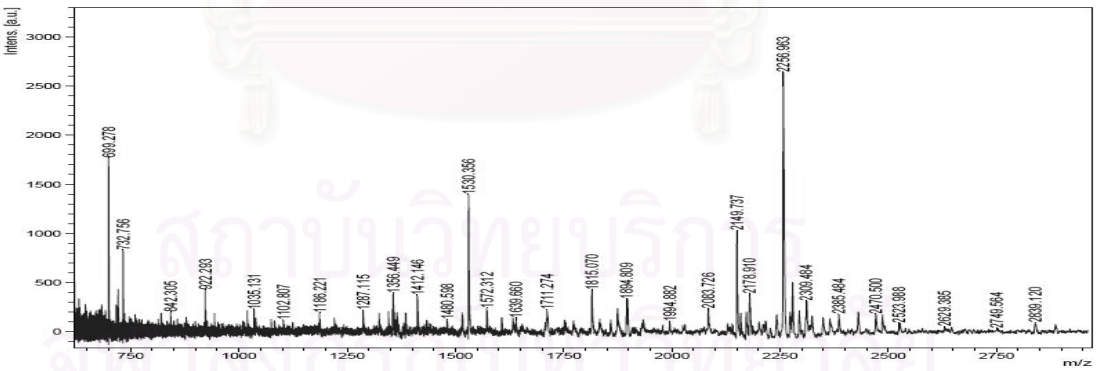

Figure 10F MALDI-TOF mass spectra of tryptic fragment of *Mycoleptodicus* sp.: A) Replicate 1, B) Replicate 2 and C) Replicate 3.

Appendix G

Table 1G Summary of percent of intensity observed by MALDI-TOF MS from *Alternaria* sp.

m/z	% Intensity			
	Replicate 1	Replicate 2	Replicate 3	Average
657.513	12.343	12.933	13.065	12.780
699.291	20.367	21.470	21.882	21.240
732.769	22.861	23.751	24.374	23.662
752.513	16.808	17.352	17.468	17.209
916.233	10.772	11.042	11.122	10.979
977.220	11.311	11.793	12.074	11.726
1412.204	15.739	16.183	16.109	16.010
1723.537	10.460	10.514	10.375	10.450
1750.835	11.573	11.499	11.105	11.392
2030.151	57.587	49.823	54.019	53.810
2085.523	11.783	10.952	10.636	11.123
2163.189	12.360	11.563	9.020	10.981
2180.686	16.821	16.348	15.849	16.339
2256.646	100	100	100	100

Table 2G Summary of percent of intensity observed by MALDI-TOF MS from Endophytic fungi sp. J48 from coffee leaves.

m/z	% Intensity			
	Replicate 1	Replicate 2	Replicate 3	Average
699.285	20.163	19.611	18.4792	19.417
732.723	17.630	16.017	15.743	16.463
801.936	15.546	14.250	13.444	14.413
1088.197	21.505	19.039	18.167	19.570
1226.382	55.237	58.487	57.961	57.229
1411.333	37.755	37.804	36.194	37.251
1418.059	19.434	17.953	18.823	18.736
1454.161	100	100	100	100
1534.308	41.442	38.740	37.565	39.249
1854.048	30.970	32.286	32.347	31.868
2057.217	24.242	22.842	23.839	23.641
2256.519	79.373	79.401	80.689	79.821

Table 3G Summary of percent of intensity observed by MALDI-TOF MS from *Bipolaris* sp.

m/z	% Intensity			
	Replicate 1	Replicate 2	Replicate 3	Average
630.655	18.892	20.136	19.999	19.675
699.244	100	100	100	100
732.734	62.614	60.009	59.290	60.638
736.726	18.138	17.842	17.753	17.911
820.565	18.121	17.298	17.220	17.546
922.283	28.120	27.809	27.718	27.882
1287.121	32.861	30.951	30.610	31.474
1354.594	36.182	41.112	40.532	39.275
1356.423	20.509	16.429	15.879	17.606
1412.136	28.925	26.828	26.497	27.417
1454.288	22.234	21.027	20.805	21.355
1480.588	20.521	17.927	17.416	18.621
1515.804	26.200	25.629	25.445	25.758
1530.333	23.664	21.815	21.409	22.296
1572.299	45.316	44.751	44.025	44.698
1758.585	25.636	23.829	22.787	24.084
1815.061	26.103	24.731	24.315	25.050
1896.814	34.504	23.844	22.582	26.977
2158.352	14.020	15.966	15.767	15.251
2178.720	14.351	16.635	15.644	15.543
2201.136	24.176	20.483	20.042	21.567
2277.877	32.857	31.914	31.544	32.105
2293.393	30.963	29.199	28.381	29.514
2470.058	22.983	22.998	21.518	22.500

Table 4G Summary of percent of intensity observed by MALDI-TOF MS from *Xylaria* sp.

m/z	% Intensity			
	Replicate 1	Replicate 2	Replicate 3	Average
699.283	20.916	17.538	23.713	20.722
732.748	12.245	10.948	13.375	12.190
1359.494	13.507	12.796	13.993	13.432
1518.725	100	100	100	100
1386.842	9.179	8.934	9.114	9.076
2042.741	27.206	30.103	25.584	27.631
2180.641	10.507	12.015	9.197	10.573
2256.652	68.668	70.503	66.226	68.466
2439.989	51.099	54.213	47.208	50.840

Table 5G Summary of percent of intensity observed by MALDI-TOF MS from *Mycoleptodicus* sp.

m/z	% Intensity			
	Replicate 1	Replicate 2	Replicate 3	Average
624.768	13.925	13.391	13.530	13.615
699.274	100	100	100	100
716.866	15.051	13.123	13.333	13.836
720.980	21.709	20.963	20.918	21.197
922.291	22.858	22.331	22.487	22.559
1019.419	10.385	11.797	11.113	11.098
1035.132	11.419	12.065	12.254	11.912
1186.228	9.929	9.658	9.909	9.832
1220.489	9.442	8.448	8.661	8.850
1287.120	11.547	11.383	11.603	11.511
1345.694	11.785	10.247	10.649	10.894
1354.628	13.383	12.248	12.544	12.725
1356.456	19.512	16.911	17.650	18.024
1384.878	10.438	9.9106	10.136	10.161
1412.162	23.484	21.464	22.276	22.408
1515.777	10.488	9.103	9.878	9.823
1530.353	88.254	78.698	80.722	82.558
1572.327	15.807	13.118	13.805	14.243
1815.060	25.101	22.864	23.274	23.746
1894.824	14.889	0.000	11.160	8.683
2178.763	18.715	14.939	15.267	16.307
2256.797	96.454	92.528	90.595	93.193
2277.932	13.285	13.391	11.734	12.803

BIOGRAPHY

Miss Benjaporn Thiensong was born on February 11, 1983 in Bangkok, Thailand. She graduated with Bachelor Degree of Science, from Department of Biology, Faculty of Science, Mahidol University in 2004. She was admitted to the Master degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University in 2004.