RECOVERY OF BACTERIAL DNA FROM SOIL WITH GROWTH OF WILD MUSHROOM

CHAN WENG HOY

THIS DISSERTATION IS PRESENTED TO FULFILL THE PARTIAL REQUIREMENT TO OBTAIN A BACHELOR OF SCIENCE DEGREE WITH HONOURS

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2.	**EXAMINER 1** **(PROF. DR. HO COY CHOKE)**	
3.	**EXAMINER 2** **(DR. VIJAY KUMAR)**	
4.	**DEAN** **(PROF. MADYA DR. AMRAN AHMED)**	

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ABSTRACT

Bacterial DNA was isolated from two types of soil sample near the growth of wild mushroom found in Universiti Malaysia Sabah campus area and were studied by using 16S ribosomal DNA (16S rDNA) assay. Two mushroom species, the *Calvatia gigantean* and another unknown mushroom species were discovered. The soil samples were undergone direct DNA extraction to recover the total DNA using a modified protocol from Zhou *et al.*, (1996). The extracted DNA was brownish in colour due to the co-extraction of humic substances. The extracted DNA was then subjected to gel electrophoresis to check for the quality of the extracted DNA while compare to the marker, Lambda *Hind*III. From the gel electrophoresis, the extracted DNA was in expected size which was bigger than 23kb. After the DNA extraction, the DNA was subjected to purification seen the present of humic substances which was the inhibitor for the polymerase chain reaction (PCR) amplification in the DNA samples. The DNA samples were purified by using DNA Grade HTP Hydroxyapatite minicolumn method which was a modified protocol from Purdy *et al.*, (1996). From the result showed in the gel electrophoresis, the concentration of first elution DNA was higher compare to second elution. Between the two DNA samples from two different types of soil sample, the first mushroom's soil purified DNA was in white colour while the second mushroom's soil purified DNA was light brown in colour. This showed that there were still humic substances contained in the second mushroom's soil purified DNA. Both purified DNA were then subjected to PCR amplification by using 16S rDNA universal primer to amplify the conserve region in the genome of microorganisms. The DNA samples were diluted in 10X, 100X and 500X to overcome the problem of inhibition causing by the humic substances. From the PCR result obtained, only the first mushroom's soil DNA sample at 10X dilution showed the amplification and the result was reproducible. While for other DNA samples, there was no amplification showed. This was mainly due to the inhibition cannot be overcome by doing dilution for second mushroom's soil sample. As the conclusion, DNA should be pure enough and in good condition in order to give a high quality of PCR product.

ABSTRAK

DNA bakteria telah diextrakkan daripada dua jenis sampel tanah berdekatan dengan kawasan pertumbuhan cendawan liar yang dijumpai dalam kampus Universiti Malaysia Sabah dan telah dikaji dengan menggunakan kaedah 16S ribosomal DNA (16S rDNA). Dua jenis spesies yang dijumpai merupakan *Calvatia gigantean* dan satu lagi yang tidak dapat dikenalpasti. Tanah yang diperolehi itu telah dijalani pengekstrakan DNA terus untuk memperoleh jumlah DNA dengan menggunakan kaedah daripada Zhou *et al.*, (1996) yang telah diubahsuai. DNA yang diperolehi adalah berwarna perang disebabkan oleh pengekstrakan bersama bahan berhumik. DNA yang diperolahi telah dijalankan dengan gel elektroforesis untuk mengetahui kualitinya apabila berbanding dengan penunjuk Lambda *Hind*III. Saiz DNA yang diperolehi adalah melebihi 23kb dan merupakan saiz yang dijangka. Oleh kerana kehadiran bahan berhumik yang merupakan perencat bagi amplifikasi tindakan rantai polimerase (PCR), penulenan DNA telah dijalankan dengan menggunakan kaedah minikolum DNA Grade HTP Hydroxyapatite yang diubahsuai daripada Purdy *et al.*, (1996). Daripada keputusan yang diperolehi, elusi DNA pertama memberikan kepekatan DNA yang lebih tinggi berbanding dengan elusi DNA kedua. Antara kedua-dua jenis sampel, sampel DNA daripada tanah pertumbuhan cendawan pertama yang ditulenkan adalah berwarna putih manakala adalah perang muda bagi DNA daripada tanah pertumbuhan cendawan kedua yang ditulenkan. Ini menunjukkan masih tertinggal bahan berhumik dalam sampel DNA. Kedua-dua sampel DNA yang ditulenkan telah digunakan untuk amplifikasi PCR dengan menggunakan primer universal 16S rDNA untuk mengamplifikasikan bahagian konserve dalam genom mikroorganisma. Sampel DNA telah dicairkan dalam 10X, 100X dan 500X untuk mengatasi masalah perencatan yang disebabkan oleh bahan berhumik. Daripada keputusan, hanya sampel DNA dari tanah cendawan pertama pada 10X memperohehi hasil manakala tidak bagi sampel DNA yang lain. Ini mungkin disebabkan oleh kesan perencatan tidak dapat diatasi dalam sampel DNA dari tanah cendawan kedua dengan kaedah pencairan. Secara kesimpulan, ketulenan dan keadaan DNA yang baik adalah penting dalam memperolehi hasil PCR yang baik.

CONTENTS

ABBREVIATION

ddH_2O	double distilled water
DNA	deoxyribonucleotide acid
RNA	ribonucleic acid
CTAB	hexadecyltrimethylammonium bromide
SDS	sodium dodecyl sulfate
EDTA	ethylenediamine tetraacetic acid
UV	ultraviolet
EtBr	ethidium bromide
PCR	polymerase chain reaction
AFLP	amplified fragment length polymorphism
RFLP	restriction fragment length polymorphism
RAPD	random amplified polymorphic DNA
RAHMS	random amplification of hybridizing microsatellites
rDNA	ribosomal DNA
rRNA	ribosomal RNA
RAMPs	random amplification of microsatellites polymorphisms
SSR	simple sequence repeat
STR	short tandem repeats
U	unit
cDNA	complementary DNA
dNTPs	deoxyribonucleotide triphosphates
ddNTPs	dideoxyribonucleotide triphosphates
TAE	Tris-Acetate EDTA

PEG	polyethylene glycol
A	adenine
T	thymine
G	guanine
C	cytosine
DGGE	denaturation gradient gel electrophoresis
DNases	deoxyribonucleases
RNases	ribonucleases

NOMENCLATURE

bp	base pairs
g	gram
µl	microliter
°C	degree Celsius
%	percent
rpm	revolutions per minute
ml	milliliter
kb	kilobase pairs
mM	milimolar
M	molar
min	minute

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

The biosphere is dominated by microorganisms (Whitman *et al.*, 1998), yet most microbes in nature have not been studied. Traditional methods for culturing microorganisms limited to those that grow under laboratory conditions (Staley and Konopka, 1985; Hugenholtz and Pace, 1996). The recent surge of research in molecular microbial ecology provides compelling evidence for the existence of many novel types of microorganisms in the environment in numbers and varieties that dwarf those of the comparatively few microorganisms amenable to laboratory cultivation (Hugenholtz *et al.*, 1998; Ward *et al.*, 1990; Giovannoni *et al.*, 1990).

The land covers ¼ of the Earth's surface. The land is consisting of many kinds of soil such as sand, rock, clay and so on. Soil is the top layer of earth in where plants grow. It is a black or dark brown material typically consisting of a mixture of organic remains, mineral particles, clay and rock. Soil is one of our most valuable resources. It regulates global biogeochemical cycles, filters and remediates anthropogenic

pollutants and the most important was the soil enables food production (Kennedy and Smith, 1995; Richards, 1987).

In the study of diverse habitat, both culture-based and culture-independent approaches support the hypothesis that soil represents one of the most diverse habitats for microorganisms (Whitman *et al.*, 1998). Molecular based studies have confirmed soil as an environment particularly rich in diversity by comparing 16S ribosomal RNA sequences from several divergent bacterial divisions. Despite these findings, the extent of microbial diversity in nature is still largely unknown. This insight provides the scientific foundation for a renewed interest in examining soil microorganisms for novel pharmaceuticals and has inspired the development of approaches to access the metabolic potential of soil microorganisms without culturing them.

However, there was not much study on the microbial communities nearby the mushroom's area that had been done. Due to the fact that there were large amount of mushroom species in this world, with a big portion of them were the toxic producer, different kinds of mushroom with different toxic such as the Ibotenic Acid, Muscarine, Muscimol, Orellanine and so on would kill the microorganisms nearby to them. However, there were some microorganisms that will still survive in those toxic conditions. As a result, the study of microbial diversity from those target area will gave researchers a better idea about the genetic make up and genes that were capable to produce protein that can be used to degrade toxic materials.

Molecular method based on 16S ribosomal DNA (16S rDNA) can be used to overcome the difficulties of bacteria culturing method. 16S rDNA was used to identify

different kinds of soil microbe. This is due to the present of 16S rDNA among various kinds of bacteria species and also bacteria from different environment (Barnes *et al.*, 1999). This approach was used in the bacteria phylogenetic analysis and domain database formation.

In this study, the total DNA extracted from soil samples at mushroom's area were amplify by using 16S ribosomal DNA primer. By using 16S rDNA analysis technique, the conserve regions of the soil microbial communities can be studied without culturing any types of bacteria from the target soil sample. This conserve region helped to characterize the microbial diversity of the microorganisms in different environments (Barnes *et al.*, 1999). As a result, the important gene for the survival of microorganisms nearby the mushroom's area can be known. From the study also, with proper development and application, useful genes can be clone into host cell in order to produce new drugs, enzymes or even medicine for the benefits of mankind.

1.2 OBJECTIVE

The main objective of this study is to recover the total DNA from the soil microbial community around mushroom's area by using 16S rDNA analysis technique. The extracted DNA was amplified by using 16S rDNA universal primer which will only amplified the conserve region in the genome of the microorganisms.

CHAPTER 2

LITERATURE REVIEW

2.1 MUSHROOM

There are several kinds of fungi are sources of human food, of which the most important are the mushrooms. Mushrooms are group of filamentous basidiomycetes belong, along with yeasts and molds, to the kingdom of Fungi in the eukaryote domain that form large and edible structures called fruiting bodies. The fruiting body is commonly called as the mushroom and is formed through the association of a large number of individual hyphae to form mycelium (Madigan *et al.*, 2003).

During most of its existence, the mushroom fungus lives as a simple mycelium, growing in soil, leaf litter or decaying logs. However, when the environmental conditions are favorable, usually following periods of wet and cool weather, the fruiting body develops, beginning first as a small button-shaped structure underground and then expending into the full-growth fruiting body that we see above ground. Sexual spores, called basidiospores are formed, borne on the underside of the fruiting body on flat plates called gills, which are attached to the cap of mushroom. The mushroom basidiospores are dispersed by wind and eventually light on a

favorable, usually moist and organic rich soil, and begin the life cycle again (Madigan *et al.*, 2003).

However, not all the mushroom are eatable or can served as food. Some of the mushroom species are toxic to human once consume or just only have lightly contact with it. These toxics include the Amatoxin, Coprin, Gyromitrin, Ibotenic Acid, Muscarine, Muscimol, Orellanine and Psilocybin. These toxins were found in several mushroom species that can cause mushroom poisoning (Madigan *et al.*, 2003).

Mushroom poisoning is caused by the consumption of raw or cooked fruiting bodies (mushrooms, toadstools) of a number of species of higher fungi. The term toadstool (from the German Todesstuhl, death's stool) is commonly given to poisonous mushrooms, but for individuals who are not experts in mushroom identification there are generally no easily recognizable differences between poisonous and nonpoisonous species. Old wives' tales notwithstanding, there is no general rule of thumb for distinguishing edible mushrooms and poisonous toadstools. The toxins involved in mushroom poisoning are produced naturally by the fungi themselves, and each individual specimen of a toxic species should be considered equally poisonous. Most mushrooms that cause human poisoning cannot be made nontoxic by cooking, canning, freezing, or any other means of processing. Thus, the only way to avoid poisoning is to avoid consumption of the toxic species (Madigan *et al.*, 2003).

Due to the fact that toxics chemical are produced naturally by the mushroom, which is the secondary metabolites, it may be capable to inhibit the growth of certain

microorganism around it by releasing the chemical compound into the surrounding environment. In this case, the study of soil microbial diversity around mushroom area and probably the mushroom secondary metabolites may help in developing new useful drugs or enzymes to cure certain diseases or even as the medicine for food poisoning faced by mankind nowadays.

Nowadays, more and more diseases are face by mankind, the search for new antiviral drugs and other enzymes to overcome the problems like tumors or cancer have become the major research and aim in modern biotechnology. All these were done by the search of the gene coding for the protein, e.g. the antiviral drugs, follow by cloning and finally set for mass production of that particular drugs that have its commercial values and helps to cure certain diseases.

2.2 MICROBIAL DIVERSITY

Microbial populations are potentially one of the greatest sources of biodiversity in soil. Some of them still not yet had been discovered (Duarte *et al.*, 1998). Many investigators reported that only 1 to 10% of these organisms are culturable on laboratory media (Duarte *et al.*, 1998). As a result, to know the microbial diversity in the soil, methods have been developed to extract the nucleic acids directly from the soil.

Due to the fact that microbial populations are one of the greatest resources of biodiversity in soil, it is rather complicated and difficult to isolate all the bacteria from the soil for the purpose of study (Trevors, 1995). Bacteria, which are adhesive on the

soil particle surface normally, have a net negative charge, resulting in repulsion at large distances (Trevors, 1995). According to the colloid stability theory, the interaction between negatively charge particles is functioning in ionic strength and in particular, the concentration of divalent or polyvalent of cations (Trevors, 1995). As a result, the direct extraction of nucleic acids from soil is necessary for the studies of microbial communities.

Microbial diversity is important in the field of biotechnology, which has become more and more important in nowadays. The discovery of new genes and biochemical processes in producing new antibiotics and enzymes had become very important for mankind to fight against diseases. Researchers nowadays have tried to discover the microbial world in those places with extreme condition like the high temperature and high salt environment. The diversity and complexity of soil microbial communities present a major challenge to our efforts to understand how biological processes can be managed in agricultural systems. Soil microbial communities are arguably the most diverse communities on earth, and the factors that determine this extraordinarily high diversity are not well understood (Caldwell *et al.* 1997). Torsvik *et al.* (1994) have provided evidence that in one gram of soil there are billions of individual organisms and thousands of species.

2.3 GENE TRANSFER IN SOIL ENVIRONMENT

The exchange of genetic information including the transformation, conjugation and transduction may occur in the soil environment and could give impact on the microbial communities studies as the gene transferred may alter the gene expression

and also will alter the bacterial metabolic activities. As a matter of fact, in the study of microbial communities, the exchange of genetic information should be considered.

2.6 Transformation

Transformation is one of the gene transfers that may occur in the microbial communities. Transformation results in the acquisition of new genetic material by the uptake of 'extracellular' DNA into a competent cell. 'Competent' is the physiological state of a cell that allows it to uptake of 'extracellular' DNA (Smith *et al.*, 1981). The recipient of the gene transfer is known as transformants (Hartwell *et al.*, 2000). Although transformation was the first identified mechanism of genetic exchange in bacteria (Avery *et al.*, 1944; Smith *et al.*, 1981), it has been considered to be a relatively unimportant mechanism for the transfer of genetic information in soil and other natural habitats, mainly because of the presumed susceptibility of 'naked' DNA to microbial degradation (Stotzky, 1989) and the difficulty to explain the presence of competent cells in natural environments.

With respect to the presence of naked DNA in soil, several studies have shown that DNA in soil can originate from numerous sources, such as lysis of dead cell due to the phage infection and the release of both plasmid and chromosomal DNA during the 'competence' phase of bacterial cells as found in *Bacillus subtitis* and *Acinetobacter calcoaceticus* (Stotzky *et al.*, 1996). Recent observation on the fate of extracellular DNA in natural habitats indicated that despite the abundance of DNases in the environment, DNA could persists as a result of its interaction with soil particles. The adsorption and binding of DNA on sand and clay minerals, such as the

montmorillonite and kaolinite, have appear to protect DNA against degradation by DNases without inhibiting it's transforming ability (Stotzky *et al.*, 1996).

2.3.2 Conjugation

Another type of gene transfer is the conjugation. In conjugation, the donor carries a special type of plasmid that allows it to come in contact with the recipient and transfer the DNA directly to the recipient. The recipient of the gene transfer is known as exconjugants (Hartwell *et al.*, 2000). Self-transmissible plasmids can mediate their own transfer since they carry both an origin of transfer (*oriT*) and transfer genes (*tra*), which are necessary for the formation of mating pairs, nicking of the plasmid and transfer of the DNA (Smit and Van Elsas, 1995). The plasmids are grouped in so called incompatibility groups and plasmids of some groups (IncP, IncN, IncW and IncX) can mobilize non-conjugative plasmids, carrying *oriT* to a recipient (Smit and Van Elsas, 1995). Recently, gene transfer in the opposite direction was detected. This process was called retro-transfer (Mergeay *et al.*, 1987). The occurrence of retro-transfer might have implications for transfer of recombinant DNA from introduced bacteria to indigenous ones. Successful conjugal transfer depends on close contact between donor and recipient cells under favorable conditions of temperature, pH and nutrient availability.

2.3.3 Transduction

In transduction, the donor DNA is packaged within the protein coat of a bacteriophage and transferred to the recipient when the phage particle infects it. The recipient of the

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