ISOLATION AND IDENTIFICATION OF CULTURABLE SOIL MICROORGANISMS FROM PORING HOT SPRING, RANAU, MALAYSIA.

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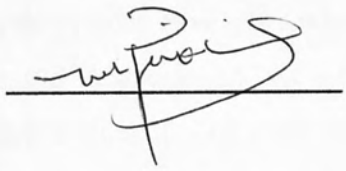
   (MS. TEOH PEIK LIN) 

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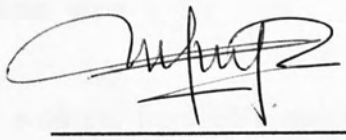
   (DR. VIJAY KUMAR) 

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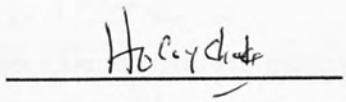
   (PROF. HO COY CHOKE) 

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ABSTRACT

Eighteen unknown microorganisms had been cultured and isolated from the soil sample in Poring Hot Spring, Ranau, Malaysia. These microorganisms were grown around 12 hours on LB agar culture medium at 50 °C. The microorganism was chosen depending on their visual morphology characteristics. After the pure culture of unknown microorganisms were obtained, the PCR reactions were carried out using forward primer, fD1 and reverse primer rP2 to amplify its 16S rRNA. The 16S rRNA of eighteen of the unknown microorganisms was examined by agarose gel electrophoresis on an ethidium bromide-stained 0.8 % agarose gel. Only nine unknown microorganisms (Unknown Microorganism 1, Unknown Microorganism 2, Unknown Microorganism 3, Unknown Microorganism 4, Unknown Microorganism 6, Unknown Microorganism 10, Unknown Microorganism 11, Unknown Microorganism 12, and Unknown Microorganism 17) were able to amplify the 16S rRNA and the size of the 16S rRNA was estimated around 1,500 bp. All nine bands were recovered by using QIAquick Gel Extraction Kit (QIAGEN, USA) after electrophoresis. Only PCR products of 4 unknown microorganisms (Unknown Microorganism 1, Unknown Microorganism 2, Unknown Microorganism 3, and Unknown Microorganism 17) were recovered successfully. However, sequencing of these fragments was unsuccessfully due to the low concentration of template DNA.

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ABSTRAK

Lapan belas microorganisma yang tidak diketahui telah dikulturkan dan diasingkan daripada sampel tanah di Poring Hot Spring, Ranau, Malaysia. Microorganisma-microorganisms ini ditumbuhkan selama 12 jam di atas kultur media LB pada suhu 50 °C. Microorganisma tersebut telah dipilih bergantung kepada sifat morphologi yang dapat dilihatkan pada satu kumpulan microorganisma. Selepas kultur tulen pada microorganisma-microorganisma yang tidak ketahui didapatkan, PCR telah dijalankan dengan menggunakan primer hadapan, fD1 dan primer belakan, rP2 untuk mengamplifikasikan 16S rRNA mereka. 16S rRNA daripada lapan belas microorganisma tersebut telah diujikan dengan menggunakan gel agarose elektroforesis pada 0.8 % agarose gel dengan pewarna ethidium bromide. Hanya sembilan microrganisma yang tidak diketahui (Microrganisma Yang Tidak Diketahui 1, Microrganisma Yang Tidak Diketahui 2, Microrganisma Yang Tidak Diketahui 3, Microrganisma Yang Tidak Diketahui 4, Microrganisma Yang Tidak Diketahui 6, Microrganisma Yang Tidak Diketahui 10, Microrganisma Yang Tidak Diketahui 11, Microrganisma Yang Tidak Diketahui 12, dan Microrganisma Yang Tidak Diketahui 17) telah dapat mengamplifikasikan 16S rRNA mereka dan saiz 16S rRNA mereka dianggarkan lebih kurang 1,500 pasangan tapak. Semua sembilan hasil PCR telah dipulihkan dengan QIAquick Gel Extraction Kit (QIAGEN, USA) selepas elektroforesis. Hanya empat microorganisma daripada hasil PCR (Microrganisma Yang Tidak Diketahui 1, Microrganisma Yang Tidak Diketahui 2, Microrganisma Yang Tidak Diketahui 3, dan Microrganisma Yang Tidak Diketahui 17) telah berjaya dipulihkan dan dihantarkan untuk tindak balas penjujukan DNA. Tetapi, empat hasil PCR telah gagal untuk tindak balas penjujukan DNA kerana kepekatan hasil pemulihan yang rendah.

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LIST OF SYMBOLS/ABBREVIATIONS

%	Percentage
°C	Degrees Celsius
µl	Micro Liter
bp	Base Pairs
cm	Centimeter
ddH_2O	Double Distilled Water
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleoside Triphosphates
kb	Kilo Base Pairs
KCl	Potassium Chloride
mg	Milligram
$MgCl_2$	Magnesium Chloride
ml	Milliliter
NaCl	Sodium Chloride
ng	Nanogram
pmol	Pico Molar
rpm	Revolutions Per Minute
SDS	Sodium Dodecyl Sulphate
Tris-HCl	Tris Hydrochloride
UV	Ultra Violet
V	Volts
w/v	Weight Over Volume

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

There are a huge number and diverse group of microorganisms that appeared in all environmental systems. These microorganisms are generally able to grow, generate energy, and reproduce. The study of these microorganisms is under one field, called "Microbiology" and this study is involved in two themes; basic and applied. As basic biological science; it provides research tools for probing the nature of life process. The understanding of the chemical and physical basic of life is proceeding from the studies of microorganisms because all cells have many in common and share many biochemical properties. As applied biological science; it have important role in medicine, agriculture, and industry. So, as unfold, microorganisms are very important to human activities and the whole web life on earth (Martigan *et al.*, 2003).

Soil and water are the rich sources of microorganisms. Depending on the location of sources, one gram of soil contains between 10^6 to 10^9 microorganisms including bacteria, filamentous fungi and yeasts, protozoa, algae, and microscopic multi-cellular forms. This shown that not only there are many microbes in soil; there are also many different species of microbes in soil. Some scientists had estimated that

one gram of soil containing 10000 different species of microorganisms (Torsvik *et al.*, 1990). However, microbiologist estimated at most 1% of soil microorganisms can be cultured and observed in laboratories because each microbial strain or species has certain nutritional and environmental requirements (Amann *et al.*, 1995). This small amount of microorganisms showed the substantial diversity and the difficulties while identifying these microorganisms. Varieties of strategies have developed to identify microbe's species accurately. Traditional identification required extensive testing that includes staining and the inoculation of selective and determinant media. But now molecular identification have developed, which include the amplification of genetic material and use of probes to detect the targeted sequences (Boyle & Albrecht, 2002).

Hot spring is one of the thermal environments that many thermophiles are present with optima range temperature in 45-80°C. In hot springs, when the boiling water overflows from the edges of the spring and flows away from the sources, the water gradually cools, and setting up a thermal gradient. Along this gradient, various microorganisms grow with different species in a temperature range. There are several types of thermophilic microorganisms, such as cyanobacteria, anoxygenic phototrophic bacteria, and chemotrophic sulfur bacteria, which differ according to the physical and chemical conditions they favor and other environmental factors. From this fact, some conclusion is made. Normally, prokaryotic organisms are able to grow at temperature that higher than eukaryotic organisms can grow. The most thermophilic prokaryotic species is *Archaea* and nonphototrophic organisms are able to grow at higher temperature if compared to phototrophic organisms (Martigan *et al.*, 2003).

A pure culture of microorganisms is necessary for the genetic studies to understand the genetic diversity of species in nature. Due to cultural difficulties, these cultivable microbes are representing only a small portion of genetic diversity of a microbial species in microbial communities and obtaining microorganisms that are representative of the diversity existing in nature can be difficult as a result of the inherent bias due to culture media. Pure culture can be obtained by the isolation method consists of serial dilution method, spread plate method, and streak dilution plate method to study morphological properties and staining reactions, cultural characteristics, physiological requirements and chemical activities, pathogenicity, and agglutination or specific reaction toward antibiotics. (Srivastava & Singhal, 1994).

The principal of serial dilution method is an original inoculum is subjected to a serial of successive dilution. The concentration of microorganisms in the serial dilution test tubes will decrease and become less and less. These microbes' suspensions are prepared for the further use of spread plate method. In spread plate method, a small volume of cell suspension is transferred from serial dilution test tube onto the surface of agar medium in a Petri plate. A sterilized inoculating loop is used to distribute the liquid over the surface of the plate. The plate is incubated and subcultured by streak dilution plate method after a period of time. In streak dilution plate method, a single colony, which containing cells of a single species and derived from a single parental cell can be achieved at the same point on the plate when a sterilized inoculating loop is used to streak the microorganism over the surface the medium to diluting the sample. An isolate is considered to be a pure stain after 3 consecutive transfers without evidence of other microorganism (Rajan, 2001; Reed *et al.*, 1998; Tate, 2000; Srivastava & Singhal, 1994).

Numerous selective and nonselective media have been used for enumerating and isolating soil and rhizosphere microorganisms. Previous work suggests that the choice of the medium has great impact on both the total count and the relative abundance of various taxonomic groups of microorganisms (Buyer, 1995). The populations of soil microorganism isolated on different media were significantly different from each other (Tabacchioni *et al.*, 2000).

Polymerase Chain Reaction (PCR) is an attractive tool for detection of specific microorganisms in microbial ecology, and effort has been devoted to the development of primers that recognize specific species. PCR is the used of replication components to amplify a specific DNA sequences and allows exponential amplification of DNA sequences that are located between two opposing primers (Burden & Whitney, 1995; Reed *et al.*, 1998). In this vitro reaction, 3 repeated steps are involved: Denaturation of DNA, Annealing of Primer, and Extension of Primer. The five essential components In PCR are template DNA, 2 primers, dNTPs, PCR buffer, and thermostable polymerase. PCR also can be used to recognize individual wild-type strains if unique sequences can be identified. Thus, PCR techniques also may be used to analyze environmental samples without culturing the microorganisms (Newton, 1995; Reed *et al.*, 1998). After the PCR reaction, the product is purified and sequencing to determine the order of the nucleotide bases along a DNA based on the principle that single-stranded DNA molecules that differ in length by just a single nucleotide can be separated from one another using polyacrylamide gel electrophoresis (Newton, 1995; Reed *et al.*, 1998).

The microorganisms are determined according to sequence analysis of gene encoding 16S rRNA (16S rDNA) that employed to assess microbial diversity (Reed *et al.*, 1998).

1.2 OBJECTIVE

The goal of research is to produce pure culture for a single cell of soil microorganisms from Poring Hot Spring in Ranau, Sabah, Malaysia. The cultirable soil microorganism is identified according to their 16S rRNA sequences. Polymerase chain reaction is uses forward primer, fD1 and reverse primer, rP2 to amplify their 16S rRNA and the PCR products is purified by using QIAquick Gel Extraction Kit (QIAGEN, USA) for direct sequencing.

CHAPTER 2

LITERATURE REVIEW

2.1 SOIL

Soil is the top layer of the earth's surface, which consisting of rock and mineral particles mixed with organic matter. Soil is formed as a result of combined physical, chemical, and biological process (Martigan *et al.*, 2003). A mature soil can be defined into 4 zones; O horizon, A horizon, B horizon, and C horizon and each horizon is different from other layers physically, chemically, and biologically or in characteristics like color, structure, texture, consistency, biotic population, and pH (Tate, 2000). The soil from the horizon that is interested is A horizon in which it is a mineral layers formed on the soil surface which below the O horizon. This region is high in organic materials associated with the mineral matter, dark in color, and is tilled for agriculture. Plants and large numbers of microorganisms grow in this horizon and the microbial activity is high here (Martigan *et al.*, 2003; Tate, 2000).

2.2 SOIL MICROORGANISM

Antonie van Leeuwenhoek was the first people whose observed bacteria just around three hundred years ago and setting up the starting point of Microbiology. Since then,

microbiology as a science has developed enormously being one of the most advanced academics among the natural sciences (Martigan *et al.*, 2003).

Soil bacteria and fungi play crucial roles in various biogeochemical cycles (BGC) and responsible for the cycling of organic compounds. Soil microorganisms also influence above ground ecosystems by contributing to plant nutrition, plant health, and soil structure and soil fertility (Trevors, 1998; Wall & Virginia, 1999).

All organisms in the biosphere depend on microbial activity. Soil microorganisms are vital for the continuing cycling of nutrients and for driving above-ground ecosystems. While many anthropogenic activities, such as city development, agriculture, use of pesticides and pollution can potentially affect soil microbial diversity. It is unknown how changes in microbial diversity can influence below-ground and above-ground ecosystems. Before changes in microbial community structure influences ecosystem functions are addressed, there is the need for reliable and accurate mechanisms of studying soil microorganisms (Kirk *et al.*, 2004).

Variety of microorganisms can be isolated from the soil environment and cultured or grown on culture medium in laboratory. Different media will encourage the growth of different types of microbes through the use of inhibitors and specialized growth substrates. Several different media can be used to isolate microbes from soil (Rajan, 2001; Srivastava & Singhal, 1994). Two common microorganisms that can be isolated and grown on the culture medium are bacteria and fungi.

Bacteria are prokaryotic microbes which have a unicellular growth form. The bacterial populations in soil are dominated by species of *Pseudomonas, Arthrobacter, Bacillus, Micrococcus, Clostridium, Achromobacter, and Flavobacterium* (Rajan, 2001; Srivastava & Singhal, 1994). Various studies had shown that approximately $4x10^3$ species of bacteria appeared in one gram of soil (Kirk *et al.*, 2004). Dilution plate techniques measure only a small portion of the total soil community but nevertheless are a useful tool for studying the relative abundance of cultivable populations and the changes in population density which occurs according to the medium used and pore forming bacteria such as *Bacillus* can be isolated by the use of their ability to withstand high temperature (Janssen *et al*, 2002; Rajan, 2001; Srivastava & Singhal, 1994).

Fungi are eukaryotic microbes with a filamentous growth form. Soil fungi are derived primarily from dilution and plating techniques. These methods are biased in favor of rapidly growing and sporulating organisms, and consequently most of the fungi identified by these techniques are Fungi *Imperfecti* (*Pencillium* and *Aspergillus*). It is extremely difficult to grow soil *basidiomycetes* on solid media in the lab, although microscopy will reveal their abundance in soil (Rajan, 2001; Srivastava & Singhal, 1994).

However, the knowledge of soil microbial diversity is limited in part by our incompetence to study these soil microorganisms. But, there is estimation that in 1 g of soil there are 4000 different bacterial "genomic units" based on DNA–DNA reassociation and that is about 5000 bacterial species have been described (Torsvik *et al*, 1990.). Approximately 1% of the soil bacterial population can be cultured by

standard laboratory practices. It is not known if this 1% is representative of the bacterial population. An estimated 1,500,000 species of fungi exist in the world. Unlike bacteria, many fungi cannot be cultured by current standard laboratory methods. Although molecular methods have been used to study soil bacterial communities, still very little research that take upon for soil fungi (Kirk *et al.*, 2004).

2.3 TEMPERATURE FACTORS AND MICROORGANISMS

Microorganisms can be grouped into categories according to their temperature ranges for growth (Martigan *et al.*, 2003).

Psychrophiles (cold-loving) are growing at 0°C, and some even as low as -10°C. Their upper limit is often about 25°C. Mesophiles are growing in the moderate temperature range, from about 20°C (or lower) to 45°C. Thermophiles (heat-loving) are growing at an optimum growth temperature of 50° or more, a maximum of up to 70°C or more, and a minimum of about 20°C. Hyperthermophiles are growing at an optimum above 75°C and can grow at the highest temperatures tolerated by any organism. An extreme example is the genus *Pyrodictium*, found on geothermally heated areas of the seabed. It has a temperature minimum of 82°, optimum of 105° and growth maximum of 110°C.

The upper temperature limit for growth of any thermophilic eukaryotic organism is about 62-65°C. And the upper limit for any photosynthetic eukaryote is about 57°C for the red alga *Cyanidium caldarium*, which grows around hot springs and has a temperature optimum of 45°C. In contrast to this, some unicellular

cyanobacteria can grow at up to 75°C, and some non-photosynthetic prokaryotes can grow at 100°C or more.

There are two major types of thermophile; the microbes that grow in geothermal sites, and those that grow in "self-heating" materials such as composts. However, some recent reports suggest that these different types of environment can share some common organisms.

Many of the prokaryotes that grow in the most extreme environments are archaea. This is a group of microorganisms that is clearly distinguishable from both the present-day bacteria and the eukaryotes. Many of them still remain to be discovered and described. The difficult field of research is the problem of reproducing their natural growth conditions in a laboratory environment. Members of the genus *Sulfolobus* (archaea) are among the best-studied hyperthermophiles (Skirnisdottir *et al.*, 2000; Martigan *et al.*, 2003). They are commonly found in geothermal environments, with a maximum growth temperature of about 85-90°C, optimum of about 80°C and minimum of about 60°C. They also have a low pH optimum (pH 2-3) so they are termed thermoacidophiles. *Sulfolobus* species gain their energy by oxidizing the sulphur granules around hot springs, generating sulphuric acid and thereby lowering the pH (Martigan *et al.*, 2003).

The study of extreme environments has considerable biotechnological potential. The two thermophilic species *Thermus aquaticus* and *Thermococcus litoralis* are used as sources of the enzyme DNA polymerase for the polymerase chain reaction (PCR) in DNA fingerprinting(Martigan *et al.*, 2003). The enzymes from these

organisms are stable at relatively high temperatures, which is necessary for the PCR process which involves cycles of heating to break the hydrogen bonds in DNA and leave single strands that can be copied repeatedly. Another thermophile species, *Bacillus stearothermophilus* (temperature maximum 75°C) has been grown commercially to obtain the enzymes used in 'biological' washing powders.

Besides that, a number of thermophiles and hyperthermophiles have been isolated from samples of hot sediments, mud, rocks, soils, and waters. Hot environments have been searched also for metal reducers. Hyperthermophiles such as *Thermotoga maritima* are known to grow as respiratory organisms when Fe (III) is provided as an electron acceptor. And *Pyrobaculum islandicum* able to reduce U (VI), Tc (VII), Cr (VI), Co (III), and Mn (IV) at 100°C (Ghosh *et al.*, 2003; Kashefi & Lovley, 2000). Sulfate-reducing bacteria (SRB), which obtain energy from dissimilatory sulfate reduction, are widespread in anoxic environments and play an important role in the sulfur cycle. Several strains belonging to the genus *Thermodesulfobacterium*, which includes thermophilic and gram-negative SRB, have been isolated from sediments and filamentous microbial communities in terrestrial hot springs (Nakagawa & Fukui, 2003).

2.4 PURE CULTURES AND MICROORGANISMS

The important step for development of microbiology was the achievement of the isolation of organisms in pure cultures. In 1872, Cohn's co-worker Joseph Schroeter had cultivated pure colonies of chromogenic bacteria, and in 1878 Joseph Lister obtained a pure culture of a milk-souring organism by dilution. Koch developed the

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