BIODIVERSITY OF THERMOPHILES IN SUNGAI KLAH AND ULU SLIM HOT SPRINGS

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A dissertation submitted in partial fulfillment of the requirements of the Degree of Master of Science (Biotechnology)

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December 2010

Specially dedicated to my loving and understanding husband, mum, dad, brother, sisters, cousins and my entire family

ACKNOWLEDGEMENTS

I thank Allah, the almighty for seeing me through my entire MSc programme.

My deepest gratitude goes to my supervisor, Dr Goh Kian Mau for his endless support and guidance throughout this project. I appreciate the words of encouragement, especially at times I was at cross-roads not knowing which way to proceed. Your guidance has immensely contributed to my intellectual development. I am very grateful and may the Almighty reward you abundantly. My appreciation also goes to Fon Lai Jing for allowing me to use some of her samples for this project.

I am grateful to my sponsors, the Islamic Development Bank for believing in my potentials and awarding me an MSc Scholarship. A big thank you also goes to Brother Lakhdarr Kadkadi and his office, at the Islamic development bank for the timely response to all our mails and requests.

I acknowledge the faculty of Biosciences and Bioengineering, for providing the research facilities. I also appreciate the help from Suhaida, Puan Fatima and all the students of Bilik Alatan Khas, especially Ummirul. I would also like to send a special thank you to my course mates and friends in UTM for their support and friendship during the course of my studies.

Finally I would like to thank my husband for trusting and allowing me to come so far away from home to study; to my mum and dad for all their love, support and prayers throughout this period. I am also very grateful to my extended family for their prayers and encouragement.

ABSTRACT

The biodiversity of thermophiles in Sungai Klah and Ulu Slim Hot Springs was determined using 16S rRNA based culture independent method. Genomic DNA was isolated from soil and water samples collected from the two hot springs. PCR amplification of the bacterial 16S rDNA was done using 27F/1492R and 27F-10/1505R-10 primer sets for conventional and mini PCR respectively. The primers A571F /U1204R were used for archaeal amplifications. TA cloning method was used. Purifed PCR products were ligated to pGEM-T cloning vector and subsequently transformed into *E.coli* DH5α. A total of 160 transformed colonies were chosen for restriction analysis by *Msp* I and *Hha* I restriction digestion. Digested plasmids were visualized by UV illumination upon 3 % gel electrophoresis and differentiated according to the DNA band patterns obtained. Sixty representative clones were initially sequenced, using T7/M13F as forward primers; this was followed by phylogenetic determination by BLAST. A total of 31 samples were sequenced using the reverse primers M13R/SP6. Sequences were assembled into full length 16S rRNA sequence using MEGA 4.1. Upon removal of sequence artefacts by Ballerophon program, 27 clones were used for phylogenetic analysis. The Sungai Klah and Ulu Slim clone sequences clustered as six main phyla; *Aquificae, Crenarchaeota, Euryarchaeota, Firmicutes, Proteobacteria,* and *Thermodesulfobacteria*. Nine clone sequences were considered unaffiliated. It can be concluded that archaea and hyperthermophilic bacteria are present in the two Malaysian hot springs studied. Sungai Klah had a richer diversity of thermophiles than Ulu Slim Hot Spring. Thermophiles belonging to all the above phyla were detected in Sungai Klah Hot Spring. Ulu Slim Hot Spring in contrast, consisted of mostly hypethermophilic bacteria and archaea. The phylum *Thermodesulfobacteria* could only be detected by Mini PCR, this phylum was not detected by conventional PCR. Thus Mini PCR broadened the scope of the sequences or phyla detected.

ABSTRAK

Biodiversiti mikroorganisma di air panas Sungai Klah dan Ulu Slim ditentukan dengan kaedah kultur independen. Genomik DNA yang telah disaring dari tanah dan sampel air dari dua sumber air panas telah diamplifikasi melalui 16S rRNA PCR. Primer yang digunakan ialah 27F/1492R dan 27F-10/1505R-10 untuk bakteria dan PCR mini. Manakala untuk bahagian archea, primer yang digunakan ialah A571F/U1204R. Pengklonanan TA digunapakai di mana produk PCR diligasi ke dalam vektor pengklonanan pGEM-T dan ini diikuti oleh transformasi ke dalam *E.coli* DH5α. Sebanyak 160 koloni dari transformasi dipilih untuk analisis pemotongan dengan enzim pencernaan *Msp* I dan *Hha* I. Plasmid yang telah dicernakan itu diperhati dalam gel elektroforesis menerusi cahaya UV dan dikategorikan mengikut pola DNA yang diperolehi. Pada mulanya, enam puluh klon telah dihantar untuk jujukan dengan menggunakan T7 / M13F sebagai primer depan. Sebanyak 31 sampel dijujukan dengan menggunakan primer songsang, M13R/SP6. Kesemua jujukan dikumpulkan untuk menjadi jujukan penuh 16S rRNA iaitu selepas penghapusan artifak PCR oleh perisian Chimera. Kemudian 27 sampel digunakan untuk analisis filogenetik. Dari kajian sekutu dari jujukan yang dianalisa, klon-klon yang diperoleh dari Sungai Klah dan Ulu Slim berkelompok menjadi enam filum utama; *Aquificae, Crenarchaeota, Euryarchaeota, Firmicutes, Proteobacteria,* dan *Thermodesulfobacteria*. Sembilan daripada sampel dianggap sebagai tiada pengkelasan. Data dari kajian ini menunjukkan kehadiran archaea dan bakteria hiperthermofilik dalam dua sumber air panas terbabit. Sungai Klah didapati memiliki kumpulan thermofilik yang lebih beraneka manakala mata air Ulu Slim mengandungi lebih banyak bacteria hiperthermofilik dan archaea. Bagaimanapun, filum *Thermodesulfobacteria* tidak dijumpai melalui proses PCR yang biasa tetapi hanya dapat dijumpai melalui PCR mini. Ini telah membuktikan bahawa PCR mini telah meluaskan lagi kajian serta skop pengenalpastian jujukan dan filum.

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LIST OF ABBREVIATIONS

^{O}C	-	Degree Celsius
%	-	Percentage
α		Alpha
β	-	Beta
bp	-	Base pair
BLAST	-	Basic Allignment Search Tool
EDTA	-	Ethylenediaminetetraacetic acid
DNA	-	Deoxyribonucleic Acid
dNTP	-	Deoxyribonucleotide triphosphate
RNA	-	Ribonucleic Acid
Hrs	-	Hours
m	-	Metre
mM	-	Milimolar
µM	-	Micromolar
Mins	-	Minute
mL	-	Mililitre
NCBI	-	National Centre for Biotechnology Information
µL	-	Microlitre

U	-	Units
PCR	-	Polymerase Chain Reaction
PO_4^{3-}	-	Phosphate
pH	-	Pressure of Hydrogen
rpm	-	Revolutions per minute
RFLP	-	Restriction Fragment Length polymorphism
Na^+	-	Sodium
K^+	-	Potassium
$MgCl_2$	-	Magnesium Chloride
X-GAL	-	5-bromo-5-chloro-2
IPTG	-	Isopopyl-β-D-1-thiogalactopyranoside
SK	-	Sungai Klah
Tris	-	Tris (hydroxymethyl) aminomethane
US	-	Ulu Slim
S	-	Seconds

CHAPTER 1

INTRODUCTION

1.1 Background

Thermophiles are microorganisms; prokaryotes (archaea and bacteria) and eukarya whose cytoplasmic membranes, proteins and nucleic acids enable them to thrive at temperatures beyond 45^{o}C (Stetter, 1996); these cellular components do not function properly at lower temperatures. Hyperthermophiles on the other hand require temperatures over 80^{o}C for optimal growth (Bauman, 2004; Stetter, 1999). Most thermophiles are prokaryotes and can survive in diverse habitats such as extremes of temperature, pH, salinity (Schlelfer, 2004) and extremes of pressure (barophiles). Biotopes for thermophiles are hot springs (Huber and Stetter, 1998; Hugenholtz *et al.*, 1998), deep sea hydrothermal vents (Holden *et al.,* 1998) and artificial hot water-containing environments such as acid mine drainage (Yin *et al.*, 2008).

The discovery of Taq polymerase from the thermophilic bacterium, *Thermus aquaticus,* in Yellow Stone hot spring in the United States triggered interest in the study of hot springs and the biodiversity within these extreme environments. The enzymes from thermophiles are of great value to the biotechnology and other industries such as

the detergent, paper bleaching, baking, brewing and molecular biology industries (Fujiwara, 2002) as well as in bioremediation. Since all naturally occurring and many man-made compounds can be degraded by microbes, studying about thermophiles will lead to the understanding and the eventual tapping of their potentials as rich sources of industrial biocatalysts (Scheifer, 2004). For example, the thermophiles present in acid mines contribute in the release of metals from their parent ores and have been used for extracting metals from low grade sulphide ores (Yin *et al*., 2008). These thermophiles have great potential; for use in the bioremediation of toxic metal wastes.

Biodiversity studies of hot springs using 16S rRNA based culture independent methods have been conducted in many parts of the world (Holden *et al.,* 1998; Huber and Stetter 1998; Huber *et al.,* 2002; Amann *et al.,* 1995). Culture independent methods are able to detect uncultivable or difficult to culture microorganism in the diversity studies of environmental samples such as those from hot springs (Baker *et al*., 2001; Isenbarger *et al.,* 2008; Kanokratan *et al.,* 2004; Schwarz *et al.,* 2007). This is because the bacteria community profiling via culture dependent approach is often over-shadowed by the fast growing and most abundant organisms in pure cultures. These fast growing microbes also known as the 'weeds of the microbial world', account for not more than 1% of the total prokaryotic population (Bauman, 2004; Hugenholtz *et al.,* 1998; Schleifer, 2004). This phenomenon is commonly referred to as the 'great plate count anomally'.

Several studies using culture independent methods have also been conducted in Asia. The biodiversity of Indonesian hot springs have been studied by Aditiawati *et al*. (2009); Aminin *et al*. (2008) and Baker *et al*. (2001). Published studies from Indonesian geothermal springs were conducting using samples collected from sites such as West Java, Domas (82 ^{o}C and 90 ^{o}C, pH 2), Cibuni (90 ^{o}C, pH 2) and Cimanggu (70 ^{o}C, pH 7), all located in West Java (Baker *et al*., 2001).; Gedongsongo hot spring (pH 6–7), Aminin *et al*. (2008) and Kawah Hujan B, at Kamojang geothermal field also located in West Java-Indonesia (Aditiawati *et al*., 2009). Kanokratan and colleagues studied the biodiversity of Bor Khlueng located in Thailand (Kanokratan *et al*., 2004). In India,

culture independent methods were used to investigate the biodiversity of Bakreshwar hot spring (Gosh *et al.,* 2003).

In Malaysia, although there are approximately 45 known thermal springs (Samsudin *et al*., 1997), a comprehensive study using culture independent methods of characterizing thermophiles, using 16S rRNA polymerase chain reaction (PCR) and other DNA based techniques is yet to be reported. Moreover, only few studies using culture dependent methods have been conducted on these hot springs. Examples of previous work on hot springs or isolation of thermophiles are a review on the potential for development of thermal springs in Malaysia (Samsudin *et al.,* 1997); the screening and identification of extracellular of lipase producing thermophiles Hamid *et al*. (2003); a nutritional study of *Aneurinibacillus thermoaerophilus*, Rahman *et al.* (2009) and L2 lipase by *Bacillus* L2 Strain (Shariff *et al.,* 2007). The most recent studies were conducted by Olusean/Akanbi and colleagues on the titles, Phenotypic and molecular identification of a novel thermophilic *Anoxybacillus* species (Olusesan *et al.,* 2009); and identification of bacilli from Selayang Hot Spring by 16S rRNA (Akanbi *et al.,* 2010). All the above studies focussed on the isolation, characterization and screening of lipase producers using culture dependent methods. .

This study sought to find out the biodiversity and relative abundance of thermopiles in two hot springs, Ulu Slim River and Sungai Klah; located in Perak, Malaysia, using culture independent methods.

1.2 Problem Statement

Interest for this research was prompted by the fact that few studies have been conducted on Malaysian hot springs and that previous studies focussed mainly on culture based methods. Since cultured microbes represent not more than 1% of the total microbial population, studying them with culture independent methods such as

comparative 16S rRNA gene analysis technique will give a more accurate view of the diversity of thermophiles in the hot springs under study.

1.3 Objectives

i. To study the biodiversity of Malaysia's hottest springs, US and SK based on the variation of the 16S rRNA gene.
ii. To determine and compare the relative abundance of thermophiles in these springs.
iii. To determine the existence of archaea from the samples analyzed.
iv. To compare results from mini PCR and conventional PCR.

1.4 Significance of the Study

This study provides 16S rRNA data on the thermophilic and hyperthermophilic bacteria and archaea present in Sungai Klah and Ulu Slim Hot Springs, using culture independent methods. This type of data was previously lacking. For the first time, the existence of archaea is reported in a Malaysian hot spring. Diverse groups of bacteria have also been detected, including unclassified phyla. If further studied, these organisms could be rich sources of thermostable enzymes in addition to other biocatalysts and proteins for industry and/or producers of secondary metabolites, useful to the biotechnological, pharmaceutical and other industries.

1.5 Scope

This study was conducted using soil and water samples collected from Sungai Klah and Ulu Slim Hot Springs. The aim of this study was to determine the biodiversity of thermophiles in Sungai Klah and Ulu Slim Hot Spring using variation in the 16S rRNA gene. This result was used to compare the abundance of thermophiles in both hot springs and to find out whether Archaea and hyperthermophiles exist in these hot springs. To achieve these objectives, DNA extracted from the samples collected from the two hot springs was amplified by PCR using both conventional and mini PCR methods. TA cloning method was used to further amplify the PCR product, extracted from the community DNA. Plasmids isolated from the positive clones were screened by RFLP and those with unique band patterns were sequenced. The resultant sequences were compared with those of GENBANK by nucleotide blast (blastn). This was followed by Chimera Check and determination of the phylogenetic affiliation of the sequences using the Naïve Bayesian Classifier on the Ribosomal Database Project release 10 website. From these results, the diversity of thermophiles in Sungai Klah and Ulu Slim Hot Spring were determined and compared. Deductions were also made on the presence of Archaea and hyperthermophiles in the two hot springs. The duration of this project was three semesters.