



Faculty of Resource Science and Technology

**ISOLATION AND CHARACTERIZATION OF THERMOPHILIC  
BACTERIA FROM ANNAH RAIS AND  
PANCHOR HOT SPRINGS**

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**Bachelor of Science with Honours  
(Resource Biotechnology)  
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Final Year Project Report

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PhD

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# Isolation and Characterization of Thermophilic Bacteria from Annah Rais and Panchor Hot Springs

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## ABSTRACT

Thermophilic bacteria are one of the most utilized bacteria in industrial sector due to their ability to withstand high temperature. Hence, by knowing the characteristic of thermophilic bacteria can boost the advancement of industries and also clinical researches. Annah Rais and Panchor hot springs are 2 known hot springs in Sarawak, Malaysia which are believed to have high numbers of thermophilic bacteria and may present some unknown species yet to be discovered. The aim of this research is to isolate and identify thermophilic bacteria in the mentioned hot springs and to screen for their potential industrial application. The methods of this research were based on the isolation and characterization of thermophilic bacteria in Annah Rais and Panchor hot springs. DNA sequences were used to identify the strains by using 16S rRNA sequencing and DNA fingerprinting. Seven isolates were successfully isolated from the hot springs and identified. Besides, they were tested with several biochemical and enzymatic production tests. In conclusion, thermophilic bacteria isolated from Annah Rais and Panchor hot springs can be further investigated for potential industrial application.

**Keywords:** Thermophilic bacteria, hot springs, isolation and characterization.

## ABSTRAK

*Bakteria termopilic merupakan salah satu jenis bakteria yang sering diguna di dalam sektor industri disebabkan bakteria tersebut dapat menahan suhu yang tinggi. Oleh itu, jika ciri-ciri bakteria termopilic dapat diketahui, ia dapat memajukan sektor industry dan dalam program penyelidikan dan pembangunan. Sarawak Malaysia terdapat beberapa air panas, and berpotensi dapat menemu bakteria yang baru and berguna. Tujuan penyelidikan ini adalah untuk mengumpul data bakteria termopilic dalam air panas yang disebutkan untuk kemas kini pangkalan data jika masa depan terdapat bakteria termopilic yang menyebabkan penyakit. Selain itu, tujuan penyelidikan ini termasuklah mencari bakteria yang berpotensi untuk menguna dalam sektor industri. Cara yang diguna dalam penyelidikan ini berasakan pengasingan dan pencirian bakteria termopilic di Air Panas Annah Rais dan Panchor. Urutan DNA akan digunakan untuk mendapat identity bakteria termopilic dengan cara penjujukan 16S rRNA. Terdapat tujuh bakteria telah dapat mengetahui identity mereka. Konklusinya, termopilic bakteria yang terdapat dalam air panas Annah Rais dan Panchor boleh disiasat untuk mengetahui applikasi dalam sektor industri.*

**Kata kunci:** *Bakteria termopilic, air panas, pengasingan dan pencirian.*

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## ABBREVIATION

<b>%</b>	<b>Percentage</b>
<b>°C</b>	<b>Degree Celsius</b>
<b>μL</b>	<b>Microlitre</b>
<b>AFLP</b>	<b>Amplified Fragment Length Polymorphism</b>
<b>AR</b>	<b>Annah Rais Hot Spring</b>
<b>Bhd</b>	<b><i>Berhad</i></b>
<b>bp</b>	<b>Base pairs</b>
<b>cm</b>	<b>Centimeter</b>
<b>CO<sub>2</sub></b>	<b>Carbon dioxide</b>
<b>comb.</b>	<b>Combination</b>
<b>CSF</b>	<b>Cerebrospinal fluid</b>
<b>CTAB</b>	<b>Cetyl trimethylammonium bromide</b>
<b>ddH<sub>2</sub>O</b>	<b>Double Distilled Water</b>
<b>DNA</b>	<b>Deoxyribonucleic Acid</b>
<b>dNTPs</b>	<b>deoxynucleotide triphosphate</b>
<b>EMBA</b>	<b>Eosin Methylene Blue Agar</b>
<b>Fe</b>	<b>Iron</b>
<b>gen.</b>	<b>Genus</b>
<b>H<sub>2</sub>O<sub>2</sub></b>	<b>Hydrogen peroxide</b>
<b>hr</b>	<b>Hour</b>
<b>mAh</b>	<b>Milliampere hour</b>

<b>MgCl<sub>2</sub></b>	<b>Magnesium Chloride</b>
<b>MHA</b>	<b>Muller-Hinton Agar</b>
<b>min</b>	<b>Minute</b>
<b>ml</b>	<b>Millilitre</b>
<b>mM</b>	<b>Milli Molar</b>
<b>MSA</b>	<b>Mannitol Salt Phenol-Red Agar</b>
<b>NA</b>	<b>Nutrient Agar</b>
<b>Na</b>	<b>Sodium</b>
<b>NaCl</b>	<b>Sodium Chloride</b>
<b>NB</b>	<b>Nutrient Broth</b>
<b>NCBI</b>	<b>National Center for Biotechnology Information</b>
<b>nov.</b>	<b>New</b>
<b>PC</b>	<b>Panchor Hot Spring</b>
<b>PCR</b>	<b>Polymerase Chain Reaction</b>
<b>QG</b>	<b>Qiagen</b>
<b>RADP</b>	<b>Random Amplified Polymorphic</b>
<b>RFLP</b>	<b>Restriction Fragment Length Polymorphism</b>
<b>rpm</b>	<b>Revolution per minute</b>
<b>rRNA</b>	<b>Ribosomal Ribonucleic Acid</b>
<b>Sdn</b>	<b><i>Sendirian</i></b>
<b>SDS</b>	<b>Sodium dodecyl sulfate</b>

<b>Sec</b>	<b>Second</b>
<b>SIM</b>	<b>Sulfur Indole Motility Medium</b>
<b>SOP</b>	<b>Standard Operating Procedure</b>
<b>sp.</b>	<b>Species</b>
<b>spp.</b>	<b>Species</b>
<b>TBE</b>	<b>Tris-Borate-EDTA</b>
<b>TE</b>	<b>Tris EDTA</b>
<b>Tris EDTA</b>	<b>Ethylenediaminetetraacetic</b>
<b>TSA</b>	<b>Tryptone Soy Agar</b>
<b>UK</b>	<b>United Kingdom</b>
<b>USA</b>	<b>United States of America</b>
<b>UV</b>	<b>Ultraviolet</b>
<b>V</b>	<b>voltage</b>
<b>w/v</b>	<b>Weight/volume</b>
<b>X</b>	<b>times</b>

## 1.0 Introduction

Thermophilic bacteria have been one of the main focuses in biotechnology field because of their biotechnological potential, industry application and in clinical researches (Rahman et al., 2004; Rabkin et al., 1985). They grow in the hot environment which have the temperature between 45 and 70 °C. Hence, it is possible to isolate them from the environment such as hot springs, deep ocean-basin core, petroleum reservoirs and deep-sea hydrothermal vents (Adiguzel et al., 2009).

According to Miquel (1888), the first research about the characterization of thermophilic bacteria, bacteria were isolated from the environment of 70 °C and they were able to form aerobic spores. Since then, many thermophilic bacteria have been characterized (Maugeri et al., 2001; Belduz et al., 2003). Most of these isolated bacteria have been characterized as members of the genera *Thermoanaerobacter*, *Thermoanaerobacterium*, and *Clostridium* (Weigel & Ljungdahl, 1981; Lee et al., 1993).

Moreover, for the past few decades, thermophilic bacteria have attracted a lot of scientists to investigate due to their high potential in industrial and clinical application in addition to scientific curiosity (Adiguzel et al., 2009). According to Weigel et al. (1985), thermophilic bacteria have been used as sources of thermostable proteins and enzymes. Besides, they have also been used as biocatalysts to convert biomass into fuel-related compound and also utilize in leaching process and waste management.

However, thermophilic bacteria have been found out to be considered as potential pathogens, because they have been isolated from clinical samples and tested. Two patients were found out to have pathogenic thermophiles isolated from their cerebrospinal fluid (CSF) and have gotten septic meningitis without the presence of other bacteria which proved that thermophilic bacteria

do cause serious illness. Hence they need to be discovered more to encounter the diseases (Rabkin et al., 1985).

Since thermophilic bacteria have been found out to have a lot of benefits in industrial sector and it is believed that they have huge potential in other sectors yet to be discovered (Marchant et al., 2002). Thermophilic bacteria in Malaysia has been a huge interest in scientists and hence, Sarawak which has multiple hot springs such as Annah Rais hot springs, Paku hot springs, and Kampung Panchor hot springs, provides good opportunity to characterize them. Characterizing the thermophilic bacteria would help in updating the database of thermophilic bacteria and it will ease the future research about their applications in multiple sectors.

The purpose of the research is to characterize thermophilic bacteria in Annah Rais and Panchor hot springs, Sarawak, Malaysia by using DNA fingerprinting, DNA sequencing, assessment of enzymatic production tests and biochemical tests.

This research is important to fulfill 3 main objectives:

1. To isolate the thermophilic bacteria from Annah Rais and Panchor hot springs.
2. To characterize the thermophilic bacteria isolated from Annah Rais and Panchor hot springs.
3. To update the database of thermophilic bacteria from the data obtained from this research.

## 2.0 Literature reviews

### 2.1 Thermophilic Bacteria

Thermophilic bacteria are microorganisms which can grow in hot temperature, and their strains have an optimum growth temperature of 65 to 70 °C, which able themselves to function and metabolize normally in hot temperature environment such as hot springs (Sigurgísladóttir et al., 1993). Besides, over the past few decades, it has enough evidence to support reclassification of thermophilic members of the genus of *Bacillus* as *Thermobacillus*, *Coprobacillus*, *Anoxybacillus*, *Sulfobacillus*, *Halobacillus*, *Salibacillus*, *Marinibacillus*, *Virgibacillus*, *Amphibacillus*, *Alicyclobacillus*, *Gracilibacillus*, *Geobacillus*, *Jeotgalibacillus*, *Breviballicus*, *Paenibacillus*, *Aneurinibacillus*, and *Ureibacillus*, regarding the data obtained from 16S rRNA sequence analysis (Rahman et al., 2004; Bae et al., 2005; Iida et al., 2005).

However, thermophilic bacteria do not depend solely on temperature, but also on other environmental parameters such as pH, energy sources, nutrients also influence the existence and diversity of these kind of bacteria. Hence, different kind of environment will give rise to different kind of thermophilic bacteria (Zeikus, 1979). Moreover, recent study by Hamid et al. (2003), had isolated extracellular lipase thermophilic bacteria from Setapak hot spring in Malaysia, which included *Bacillus stearothermophilus*, *Bacillus coagulans*, *Bacillus subtilis* and *Bacillus thermoglucosidasius*.

### 2.1.1 *Geobacillus* genus

*Geobacillus* was recently split from the genus *Bacillus*, and it is phylogenetically and physical make up coherent genus of thermophilic bacilli with a similarity of 98.5 to 99.2% in 16S rRNA sequence (Nazina et al., 2001). Besides, as stated in the report of McMullan et al. (2004), this genus has been isolated from both marine and terrestrial environments. The environments included geothermal areas, temperate regions and permanently cold habitats, which indicate the adaptation of *Geobacillus* in wide variety of environmental niches. Moreover, this species has also been utilized as source of various thermostable enzymes (McMullan et al., 2004).

*Geobacillus* spp. are able to grow at the temperature of 40 to 70 °C and appeared to be aerobic or facultative anaerobic bacilli (Nazina et al., 2001). These bacteria can ferment sugars such as pentose and hexose and produce ethanol, lactate, formate and acetate as products. Moreover, *Geobacillus* spp. deficient in lactate dehydrogenase has shown high efficiency in ethanol production (Shama, 1987; Payton & Hartley, 1985; Martin et al., 1994; Javed, 1993; Thomson, 2003; Gialamas, 2006; Taylor, 2007). On the other hand, *Geobacillus zalihae* sp. nov., has been found out from palm oil mill effluent in Malaysia which is a lipolytic thermophilic bacteria which can break down lipids (Rahman et al., 2007).

### 2.1.2 *Anoxybacillus* genus

According to Dulger et al. (2004), genus *Anoxybacillus* is separate from the genus *Bacillus*. From other previous studies, this genus appeared to be different from other thermophilic bacilli in term of the utilization of sugar xylose, and one of the species that has great xylose utilization power is *Anoxybacillus gonensis* sp. nov (Belduz et al., 2003). Moreover, in the study by Pikuta et al. (2000), the *Anoxybacillus pushchinensis* gen. nov., sp. nov., is one of the bacteria which

can live at alkaline condition, also known as alkaliphilic bacilli. Moreover, another *Anoxybacillus* species is found out to be closely related genetically to *Bacillus flavothermus* (Heinen et al., 1982), which is *Anoxybacillus flavithermus* comb. nov., which is also alkaliphilic and appeared to be moderate thermophilic. Recent study of Chai et al. (2011) has reported that pullulan-degrading *Anoxybacillus* species have been isolated from Sungai Klah and Dusun Tua hot springs in Malaysia.

### **2.1.3 *Thermoanaerobacter* genus**

According to Weigel and Ljungdahl (1981), *Thermoanaerobacter* is one of the main genera of thermophilic bacteria. They are usually found in hot springs and other high temperature environment such as Yellowstone National Park. Moreover, *Thermoanaerobacter* species such as *Thermoanaerobacter brockii* has been isolated from the Yellowstone National Park hot springs (Zeikus et al., 1979), and *Thermoanaerobacter italicus* from a thermal spa in Italy (Kozianowski et al., 1997). And recently, there are few other species reported from Chinese hot springs. One of the *Thermoanaerobacter* isolated from China is *Thermoanaerobacter tengcongenesis* (Xue et al., 2001).

Besides, *Thermoanaerobacter* genus is of great biotechnology importance in the production of ethanol due to its ability in production of high yield of ethanol, by simultaneous fermentation of pentose and hexose (Hemme et al., 2011). Next, this genus is very capable in converting both pentose and hexose to ethanol and this species is very stable in when heated. However, the yields of ethanol production are varied among the species, which some of the *Thermoanaerobacter* such as *Thermoanaerobacter* sp. X514 produce more ethanol (Hemme et al., 2011). However, there is still no report about this genus found in Malaysia's hot springs.

#### **2.1.4 *Clostridium* genus**

*Clostridium* genus is thermophilic and it is able to sustain high temperature and low pH due to its morphology, the present of endospores. This species is often associated with diseases, Borriello (1995), stated that *Clostridium* is one of the most potent collections of toxigenic bacteria. Moreover, according to Songer (1996), *Clostridium* genus is widely known as pathogen to human, domestic animals and wildlife. According to Borriello (1995), *Clostridium difficile* will cause diarrhea and pseudomembranous colitis (PMC).

The most common species of *Clostridium* is *Clostridium perfringens*, it is pathogenic and causes clostridial enteric disease in domestic animals. There are few types of *C. perfringens*, such as type A, B, C, D, and E. Type A of *C. perfringens* are consistently recovered from both the environment and the intestinal tract of the animals, but the other types are less commonly found in the intestinal tract of the animals but can be found in the environment occasionally (Songer, 1996). Moreover, the other species of *Clostridium* is *Clostridium botulinum*. *C. botulinum* will produce neurotoxins. The neurotoxins can inhibit the release of neurotransmitter such as acetylcholine from motor neurons. Hence, it will cause nervous system to malfunction (Welch et al., 2000). On the other hand, there is still no *Clostridium* species been reported isolated from Malaysia hot springs.

#### **2.1.5 *Thermoanaerobacterium* genus**

*Thermoanaerobacterium* genus contains bacteria which are thermophilic, obligate anaerobes which utilize itself in carbohydrate and polysaccharide fermentation, and the products are primarily L-lactic acid, ethanol, acetic acid, carbon dioxide and hydrogen gas (Lowe et al., 1993; Lynd et al., 2002; Wiegel et al., 1985). The majority of characterized *Thermoanaerobacterium*

strains have been collected from the hot springs and some of them are from deep surface environments (Lee et al., 1993; Liu et al., 1996; Shao et al., 1995). According to Dotzauer et al. (2002), it has been found out that canned foods also do contain *Thermoanaerobacterium*.

According to Shaw (2010), some of the genus's strains are naturally competent, which they are able to amend or undergo genetic manipulation for industrial application. For example, strains *Thermoanaerobacter ethanolicus* JW200 and *Thermoanaerobacterium aotearoense* DSM 10170 are naturally competent. However, this genus has not yet been found out in Malaysia hot springs.

## **2.2 Hot Springs in Sarawak, Malaysia**

Hot springs are one of the geothermal reservoirs which have the appearance of deeply circulating fluids. According to Samsudin et al. (1997), Malaysia has about 45 hot springs, which include the current research Annah Rais and Panchor hot springs in Sarawak. The statistics of the research have shown that the 2 hot springs mentioned have a medium temperature of 43 °C. The hot springs are found in Bau, Sarawak and have a high flow rate but poor accessibility to people since they are located in the rural area of Sarawak (Samsudin et al., 1997). Besides, according to Chin et al. (2014), Annah Rais Long house which is located near to the Annah Rais and Panchor hot springs, Sarawak are recreational sites in Sarawak. However, they are in the rural area and hence it is yet to be developed and not many tourists visited the place.

### **2.3 DNA fingerprinting**

The DNA fingerprinting technique is invented by Alec Jeffreys, and when he started the technique, it soon became as a new valuable forensic tool for identification and analysis of criminal DNA (Lewin, 1989). According to Vos et al. (1995), DNA fingerprinting can display a set of DNA fragments from a desired DNA sample.

Besides, DNA fingerprinting is a powerful tool with high precision. It is able to link the hair, semen or blood at the crime scene to a person's DNA. In other words, the suspect's DNA is can be analyzed together with the blood collected at the crime scene (Roberts, 1992).

Moreover, with the presence of DNA fingerprinting, this techniques' evolution had a huge increase in the past decades (Tyler et al., 1997). A DNA fingerprinting technique, Amplified Fragment Length Polymorphism (AFLP) is used in the bacterial genome fingerprinting (Janssen et al., 1996). The technique involves 3 steps, which include the digestion of the sample's DNA with 2 restriction enzymes and ligation with specific adaptors to the fragments. Next, the selective amplification of these fragments will be done with 2 Polymerase Chain Reactions (PCR) primers. Lastly, the products will be analyzed using electrophoresis.

Besides, PCR- RFLP (Restriction Fragment Length Polymorphism), is also a DNA fingerprinting technique for bacterial identification. According to Ruiz et al. (2000), PCR-RFLP analysis is a useful tool for identification and characterization of microorganisms regarding their phylogenetic relationship, and they have demonstrated the use of RFLP in prokaryotic analysis in the year 2000.

### **2.3.1 (GTG)<sub>5</sub> DNA fingerprinting**

The oligonucleotides probe (GTG)<sub>5</sub>, is originate from the oligonucleotides that hybridized to simple repetitive DNA patterns that tare highly informative, which can act as a probe for DNA fingerprinting. This probe can be used to investigate a large variety of organisms included bacteria, fungi, plants, animals, even human (Beyermann et al. 1992). Besides, as stated in Meyer et al. (1993), this probe is usually used as a primer to detect macrosatellite DNA sequences of the organism in a PCR amplification. Moreover, according to Beyermann et al. (1992), oligonucleotide probe (GTG)<sub>5</sub> was used to detect the differences in the patterns of DNA fingerprint in plant species such as *Triticum aestivum*, *Secale cereale*, *Hordeum vulgare*, *Beta vulgaris*, *Petunia hybrida*, *Brassca oleracea*, and *Nicotiana tabacum*. Furthermore, as explained in the report of Matsheka et al. (2005), this probe can generate highly discriminatory, epidemiological valuable data for a random amplified polymorphic DNA (RAPD) assay of *Campylobacter concisus* strains.

### **2.4 16S rRNA sequencing**

16S rRNA gene has been used for identification of prokaryotes. Besides, the 16S rRNA gene is the primary key for any phylogeny-based identification (Kim, 2012). Besides, 16S rRNA sequence analysis is an intense tool for identifying new bacteria, and it is useful in clinical research especially helping the discovery of new clinical pathogens in patients. This technique is applied in the clinical laboratory to identify bacterial isolates (Patel, 2001).

Moreover, according to Patel (2001), the use of 16S rRNA gene sequences is the most common housekeeping genetic marker used, dues to they are present in almost all bacteria and usually show as a multigene operons or family. Next, the 16S rRNA's function does not vary over the

time. Lastly, the gene sequences contain around 1,500 base pairs (bp) which is large enough for informatics purposes in term of identify of the bacteria species.