UNIVERSITI PUTRA MALAYSIA

# PARTIAL CHARACTERISATION AND SOME PROPERTIES OF A THERMOPHILIC BACTERIUM ISOLATED FROM A PETROLEUM RESERVOIR IN SARAWAK, MALAYSIA

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**By**

**YOU LI LING**

**Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Food Science and Biotechnology Universiti Putra Malaysia**

**July 1999**

**Specially dedicated to :**

**My beloved**

**Daddy, Mummy and Brother,**

**Husband,**

**And Friends.**

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## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| OD | - | optical density |
| $N_2$ | - | nitrogen |
| $CO_2$ | - | carbon dioxide |
| $O_2$ | - | oxygen |
| K | - | potassium |
| Ca | - | calcium |
| Mg | - | magnesium |
| H | - | hydrogen |
| S | - | sulphur |
| $H_2S$ | - | hydrogen sulphite |
| Na | - | natrium |
| Cl | - | chlorin |
| P | - | phosphorus |
| nm | - | nanometer |
| µm | - | micrometer |
| °C | - | degree celcius |
| mL | - | mililiter |
| M | - | molar |
| Eh | - | reduction potential |
| E'o | - | standard reduction potential |
| SEM | - | Scanning Electron Microscopy |
| TEM | - | Transmission Electron Microscopy |
| UV | - | ultraviolet |
| MEOR | - | microbial enhanced oil recovery |

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science.

**PARTIAL CHARACTERISATION AND SOME PROPERTIES OF A THERMOPHILIC BACTERIUM ISOLATED FROM A PETROLEUM RESERVOIR IN SARAWAK, MALAYSIA**

By

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**July 1999**

**Chairperson : Raha Abdul Rahim, Ph.D.**

**Faculty : Food Science and Biotechnology**

The hyperthermophilic bacteria (MRU5) was isolated from an oil-producing well in Sarawak Malaysia. MRU5 is strictly anaerobic and was grown in enrichment media which were been degassed with $CO_2$ : $N_2$ (80 : 20). By Gram and spore staining, MRU5 was found to be a gram negative, non-spore producing bacteria. Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) revealed the shape of the bacteria to be irregular and coccoid which occurs singly, in pairs or clumps. The bacteria measures 0.75 μm in diameter and is surrounded by an outer sheath coating. MRU5 was found to grow best at pH 7 and 10% salinity with the optimal temperature range of between 90°C to 100°C. MRU5 was grown in xylose, glucose, fructose, arabinose, lactose, and sucrose. From the results obtained, xylose was the most efficiently utilised fermentable carbon source. Growth of the microorganism is supported by yeast extract. End products such as organic acids and $H_2S$ were produced by fermentation of carbon sources but none of

the tested solvents were produced. This microorganism is able to reduce oil viscosity by producing organic acids. This means that MRU5 has the potential to be ecologically important for enhancing oil recovery. The modified Gompertz equation was found to be appropriate to model the growth of bacterium MRU5 at different medium compositions and culture conditions.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan bagi mendapat Ijazah Master Sains.

**PENCIRIAN SEPARA DAN SIFAT-SIFAT BAKTERIA THERMOFILIK YANG DIPENCILKAN DARI TELAGA MINYAK DI SARAWAK, MALAYSIA**

Oleh

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**Julai 1999**

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Bakteria MRU5 merupakan sejenis bakteria hiperthermofilik yang disaring daripada salah sebuah telaga minyak di Sarawak, Malaysia. MRU5 adalah bakteria anaerobik, dan ditumbuhkan dalam media yang telah diperkayakan dan dinyahoksigen dengan menyalurkan campuran gas dalam nisbah $CO_2$ : $N_2$ (80 : 20). Penggunaan "Scanning Electron Microscopy" (SEM) dan "Transmission Electron Microscopy" (TEM) telah menunjukkan bakteria ini berbentuk kokus tidak sekata yang diliputi oleh lapisan selaput tebal, berdiameter 0.75 μm, dan ia wujud secara individu, berpasangan atau berkelompok. MRU5 didapati tumbuh pada keadaan optimum pada suhu 90°C, pH 7 dan dengan darjah kemasinan 10%. MRU5 ini boleh tumbuh dalam media yang mengandungi xylosa, glukosa, fruktosa, arabinosa, laktosa dan sukrosa. Keputusan menunjukkan xylosa dan estrak yis masing-masing

adalah sumber karbon dan nitrogen yang paling sesuai. Produk akhir yang dibebaskan daripada proses fermentasi sumber karbon adalah asid organik dan hidrogen sulfida. Asid organik yang dihasilkan berupaya mengurangkan kelikatan minyak. Ini menunjukkan MRU5 mempunyai potensi dalam meningkatkan pengeluaran minyak dari telaga minyak. Persamaan Gompertz yang diubahsuai adalah didapati sesuai digunakan sebagai model pertumbuhan bagi bakteria ini dalam komposisi media dan keadaan pertumbuhan yang berlainan.

# CHAPTER I

# GENERAL INTRODUCTION

Temperature is one of the most important variable in the environment. The classification of living microorganism based on their relation to temperature has therefore always been considered as one of the most basic elements of systematic biology. Microorganisms have traditionally been divided into three main groups in this respect, e.g., psychrophiles (-3°C to 20°C), mesophiles (13°C to 45°C) and thermophiles (42°C to 100°C or more).

Thermophilic microorganisms, which have an optimum temperature of more than 45°C, are found in many diverse habitats, such as thermal waters, soils and fermenting hay, hot water boilers and thermophilic biogas plant (Brock, 1978). The ranges of thermophiles still need to be divided further, and a relatively simple division is to define thermophilic and extremely thermophilic prokaryotes as those which can grow from 55-60°C to 80-85°C and hyperthermophiles as those which can grow optimally above 80-85°C. This division will put all known thermophilic bacteria (except Thermotogales) in the thermophilic range and the majority of the thermophilic archaebacteria is in the hyperthermophilic range. Thermophiles, which have been of interest for long time, both to scientists and to the public. This is understandable since these microorganisms can live and thrive under extreme conditions which most living microorganisms cannot tolerate. Thus, these microorganisms may contain extracellular and intracellular compounds such as special enzymes which are thermostable.

Thermophiles are believed to have a great potential in biotechnology and some products are already on the market. Thermostable DNA polymerase, for example, which was isolated from *Thermus aquaticus* are essential for the well-known PCR technology (Edwards, 1990).

Many enzymatic and microbiological industrial processes are carried out at high temperatures and those that are not could, in many cases, benefit from increased heating if not prevented by some unstable component in the process. The main advantages of increased temperature are generally; higher reaction rates, higher solubility of most chemicals, and increased fluidity and diffusion rates. The early notion that high temperatures prevents microbial contamination is only partly correct. It does, in fact, prevent growth of most environmental mesophilic microorganisms and pathogens but thermophilic microorganisms, especially spore formers, are surprisingly common contaminants in many fermentation processes which are run at elevated temperature.

Most of the microorganisms which have an ability to survive in oil-producing well can tolerate extreme environments. The survival of hyperthermophilic and hyperhalophilic microorganisms isolated from an oil-producing well is usually due to maintenance under strict anaerobic conditions. Various types of bacteria such as hydrocarbon-utilising bacteria and methane forming bacteria isolated from an oil producing well have several features with potential in enhancing oil recovery. The potential useful products of Microbial Enhanced Oil Recovery (MEOR) metabolism from these microorganisms are categorised into five general classes : polymers, biomass, organic acids, gases, and biosurfactants and solvents. Among those microorganisms, the most interesting microorganisms which contribute a lot in enhancing oil recovery are the fermentative bacteria. Direct utilisation of the fermentative bacteria on the sulphate and carbonates might promote the release of oil from those material. The fermentative bacteria produced acids which promoted the dissolution of carbonate rock and thus released the droplets of entrapped oil. Some of the bacteria also produced gases, including carbon dioxide, hydrogen and methane which increased the pressure and expel oil from the microtraps in oil reservoir.

Several hyperthermophilic bacteria have been isolated in Malaysia (Madihah *et al.*, 1996). A strain isolated from an oil-producing well in Sarawak was used in this study.

This project is carried out to achieve the following objectives:

i) To characterise a thermophilic bacterium isolated from an oil-producing well in Malaysia.

ii) To study the effect of each nutrient component in medium formulation and culture condition on growth of the isolated strain, and production of some metabolites such as organic acids and solvent.

iii) To study the growth kinetics of the bacterium at different medium formulations and culture conditions.

# CHAPTER II

# LITERATURE REVIEW

## Anaerobic Bacteria Adapted to Environmental Stress

A strong thread of incentive has dominated the search for new thermophilic archaebacteria, the incentive to define experimentally the maximum temperature capable of supporting life. A steady rise in the maximum temperature for bacterial growth reflects the increased interest of the scientific community together with improvement in laboratory technique and sampling technology. Table 1 shows the historical development of thermophilic microbiology (Cowan, 1992).

Table 1 : Historical Development of Thermophilic Microbiology

| Period | Growth temperature range (°C) | Representative microorganisms |
|---|---|---|
| Pre-1960s | 37-65 | *Bacillus stearothermophilus* |
| 1960s-70s | 60-85 | *Bacillus caldolyticus* |
| | | *Thermus* spp. |
| | | *Thermoplasma acidophilum* |
| | | *Sulfolobus* spp. |
| 1980s | 85-105 | *Desulfurococcus* spp. |
| | | *Thermoproteus tenax* |
| | | *Pyrodictum* spp. |

Prokaryotic microorganism can differ from eukaryotic cells in part because they have adapted to grow under extreme temperatures (>100°C), salinity (saturated NaCl), pH (<2.0,>10), and substrate stress (limited chemical free energy or on toxicants). The origin of physiological biochemistry is described as follows; pH, neutral; temperature 37°C; atmosphere, aerobic; salinity, 1.5%; substrate, glucose. Table 2 shows the comparison of requirements for optimal growth of anaerobes adapted to extreme environmental conditions.

Table 2 : Comparison of Requirements for Optimal Growth of Anaerobes Adapted to Extreme Environmental Conditions

| Class | Growth requirement | Species example | Reference |
|---|---|---|---|
| Thermophile | High temperature (>60°C) | *Thermoanaerobacter brokii* | Zeikus *et al.*, (1979) |
| Halophile | High salinity (≥10% NaCl) | *Haloanaerobium praevalens* | Zeikus *et al.*, (1979) |
| Acidophile | High acidity (external pH ≥ 2.0, internal pH<6.0) | *Sarcina ventriculi* | Smit, (1933) |
| Akaliphile | High alkalinity | *Methanohalophilus zhilinae* | Mathrani, *et al.*, (1988) |
| Syntroph | Derives limited free energy and forms inhibitory catabolites (<10 kJ/mol of substrate consumed; $H_2$, HCOOH, acetate formed), grows with a metabolic partner | *Syntrophospora bryantii* | Zhao, *et al.*, (1990) |
| CO utilizer or dehalogenator | Derives energy from substrate detoxification (CO as energy source) | *Butyribacterium methylotrophicum* | Lynd, *et al.*, (1982) |

The starting materials for synthesising the organic matter under anoxic conditions included $H_2S$, CO, HCN which are poisonous gases for most aerobic microorganisms, but these are important metabolites for certain anaerobic bacterial species. An assumption has been made that among microorganisms living today, obligate anaerobic bacteria are the simplest in structure and biochemistry, and are the most closely related to the earliest forms of life. In general, the physiological processes for adaptation to environmental stress in anaerobic bacteria seem to have involved differently from those in aerobic bacteria for two major reasons. First, anaerobes are energy limited during the chemoorganotrophic growth mode bacause they cannot couple dehydrogenation reactions to oxygen reduction and gain high level of chemical free energy (Thauer *et al.*, 1977). Second, growth of most chemoorganotrophic anaerobes (except for methanogens) is naturally associated with the generation of toxic end product (e.g., organic acids or alcohols , $HS^-$), which requires anaerobic species to develop certain dynamic adaptation mechanism or tolerance towards their catabolic end products.

## Thermophiles

A number of natural habitats of high temperatures exist, ranging from sun-heated soils and littered with temperatures of 60°C to 70°C to erupting volcanoes, reaching 1000°C (Brock, 1967). Liquid-water environments with the highest known naturally occurring temperature is close to sea level, hence had boiling points around 100°C. Over the past two decades, habitats have been found at the bottom of the ocean with temperature up to 350°C, raising questions about the possibility of life in these environments.

Thermophilic aerobic and anarobic sporeforming bacteria were isolated from self-heating soil environments before the 1940s (Brock, 1986). About two decades ago, non-sporulating aerobic bacteria were detected in thermal springs of constantly high temperature, and the first thermophilic microorganism *Sulfolobus acidocaldarius*, an archaebacterium with optimum temperature of above 80°C and upper growth temperature at around 92°C, was isolated (Brock *et al*., 1972). Another archaebacterium, *Methanobacterium thermoautotrophicum*, was the first nonsporulating thermophilic anaerobe described (Zeikus *et al*., 1972).

The diversity and ecology of thermophilic anaerobes in thermal spring environments was first described in 1979 (Zeikus, 1979 ; Zeikus, *et al*., 1979). Since then, hyperthermophilic anaerobes have been isolated from continental and submarine volcanic areas, such as solfatara fields, geothermal power plants, and geothermally heated sea sediments and hyperthermal vents (Stetter, 1986 ; Stetter and Zillig, 1985). Table 3 outlines the sites from which thermoanaerobes have been isolated. Interestingly, some microorganisms have been isolated from areas with temperature much higher than their maximum growth temperature, e.g., *Hyperthermus butylicus* and *Fervidobacterium islandicum*, which susgests that in these environments the microorganisms may not actively growing. The same