UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION, GROWTH KINETICS AND CELL DISINTEGRATION OF MRU5, A BACTERIUM ISOLATED FROM AN OIL WELL IN SARAWAK, MALAYSIA

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By

LEE SOOK FONG

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

December 2007

Specially dedicated to,
My Family, Kok Mun, Teddy and Friends…..

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

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December 2007

Chairman : Associate Professor Lai Oi Ming, PhD

Faculty : Biotechnology and Biomolecular Sciences

A hyperthermophilic bacterium, coded MRU5 was isolated from an oil-producing well in Sarawak, Malaysia. This microorganism was found to be a strictly anaerobic sulphate-reducing bacteria. Since this bacteria can survive and carry out its functions at extremely harsh conditions (high pressure, high temperature, high salinity), it is postulated that the enzymes isolated would be highly suitable for use in industrial processes, which need enzymes to be robust and stable. In this study, the cell disruption methods of the bacterium were optimized and the activity of possible industrial thermostable extracellular enzymes such as xylanases, lipases and amylolytic enzymes were determined. The bacterium, has an irregular coccoid shape, is Gram negative, and is able to grow at 90^{o}C, pH 7.0 and high salinity (10% NaCl). It is resistant to antibiotic such as ampicillin, chloramphenicol, kanamycin, rifampin, streptomycin, tetracycline and geneticin. Based on this profile, MRU5 is suggested to be a new species under the halothermophile Archaea family. 16S rRNA sequencing failed to identify the taxanomy because of limitation of universal PCR primers. Amylase, xylanase and lipase had been isolated extracellularly with the

activity of 11.8 U/L, 82.1 U/L and 7.61 U/L, respectively. Amylase was chosen for cell breakage studies as an indicator. Different methods of cell breakage were applied to obtain a high intracellular enzyme activity. The methods used were bead miller, lysozyme and combinations of bead mill with ultrasonicator and high pressure homogenizer. The breakages of cells were observed under scanning electron microscope. Amylase activity was detected intracellularly as well as on the membrane. Cell disruption treatment with only bead mill with 6g of glass beads recorded the highest specific enzyme activity (1.81 U/mg) at hour 3 where it is almost double compared to combination of bead mill and ultrasonication (0.983 U/mg) at similar time.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGENALAN, KINETIK PERTUMBUHAN DAN PEMECAHAN SEL BAGI MRU5, SEJENIS BAKTERIA YANG DIASINGKAN DARI TELAGA MINYAK DI SARAWAK, MALAYSIA

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Sejenis hipertermofilik bakteria yang dikodkan MRU5 telah berjaya diasingkan daripada telaga minyak di Sarawak, Malaysia. Mikroorganisma ini didapati tergolong dalam kategori anaerobik penurun sulfat. Bakteria ini boleh hidup dalam keadaan yang drastik seperti tekanan, suhu dan dalam tahap kemasinan yang tinggi. Oleh itu, adalah dijangkakan bahawa enzim yang terhasil amat sesuai digunakan dalam industri yang memerlukan enzim yang tahan lasak dan stabil. Objektif kajian adalah untuk mengoptimakan kaedah pemecahan sel bakterium dan menentukan aktiviti extrasellular enzim termostabil yang diperolehi seperti xilanase, lipase dan amilase. Bakteria ini berbentuk kokoid, Gram negatif, dan boleh hidup pada suhu 90^{o}C, pH 7.0 dan kemasinan yang tinggi (10% NaCl). Ia rintang terhadap antibiotic seperti *ampicilin*, *chloramphenico*l, *kanamycin*, *rifampin*, *streptomycin*, *tetracycline* dan *geneticin*. Daripada profil yang diperolehi, dicadangkan MRU5 sebagai satu spesis baru yang tergolong dalam famili Halotermofilik Archaea. Kaedah penjujukan 16S rRNA gagal mengenal pasti taksonomi MRU5 kerana kewujudan

universal primer PCR yang terhad. Amilase, xilanase dan lipase telah dihasilkan secara ekstra-selular dengan aktiviti masing-masing sebanyak 11.8 U/L, 82.1 U/L dan 7.61 U/L. Enzim amilase dipilih untuk kajian pemecahan sel sebagai penanda. Pelbagai kaedah pemecahan sel telah dilakukan untuk memperoleh enzim dan aktiviti intra-selular yang tinggi. Kaedah yang digunakan termasuklah *bead miller*, enzim *lisozyme* dan kombinasi *bead miller* dengan *ultrasonicator* dan homogenizer bertekanan tinggi. Sel yang telah pecah dilihat di bawah mikroskop elektron. Aktiviti amilase telah dikesan secara intraselular dan juga pada lapisan membran. Kaedah pemecahan sel menggunakan *bead miller* dengan 6g bebola kaca mencatatkan nilai enzim aktiviti spesifik yang tertinggi (1.81 U/mg) pada jam ketiga, di mana nilai ini adalah hampir dua kali ganda berbanding dengan kombinasi *bead miller* dan *ultrasonication* (0.983 U/mg) pada masa yang sama.

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I certify that an Examination Committee has met on **3 December 2007** to conduct the final examination of Lee Sook Fong on her Master of Science thesis entitled 'Identification, Growth Kinetics and Cell Disintegration of MRU5, A Bacterium Isolated From An Oil Well in Sarawak, Malaysia' in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

LEE SOOK FONG

Date : 31 March 2008

TABLE OF CONTENTS

		Page
DEDICATION		ii
ABSTRACT		iii
ABSTRAK		v
ACKNOWLEDGEMENTS		vii
APPROVAL		viii
DECLARATION		x
LIST OF TABLES		xiii
LIST OF FIGURES		xiv
LIST OF PLATES		xv
LIST OF ABBREVIATIONS		xvi
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	4
	2.1 Identification of Hyperthermophilic Microorganisms	4
	2.1.1 Taxonomy and Phylogenetic Relation of Hyperthermophiles	4
	2.1.2 Methods of Identification	6
	2.1.3 Archaea	8
	2.1.4 Halophilic Bacteria	10
	2.1.5 Adaptability of Thermophiles	13
	2.2 Growth Kinetics of Hyperthermophiles	14
	2.2.1 Kinetic Analysis of Hyperthermophiles	14
	2.2.2 Effect of Antibiotics	16
	2.2.3 16S rRNA Sequencing	18
	2.2.4 Thermostable Enzymes	19
	2.3 Cell Disintegration of Hyperthermophiles	31
	2.3.1 Methods of Cells Breakage	31
	2.3.2 Kinetic Analysis of Cell Disintegration of Hyperthermophiles	35
3	**MATERIALS AND METHODS**	36
	3.1 Materials	36
	3.2 Media	37
	3.2.1 Media Preparation	37
	3.2.2 Media supplements	38
	3.3 Methodology	40
	3.3.1 Identification of MRU5	40
	3.3.2 Growth Kinetics of MRU5	46
	3.3.3 Methods of Cell Disintegration of MRU5	53
4	**RESULTS AND DISCUSSION**	58
	4.1 Identification of MRU5	58
	4.1.1 Characteristics of MRU5	58

4.2 Growth Kinetic of MRU5 67
4.2.1 Growth Profiles of MRU5 67
4.2.2 Enzyme Activities of MRU5 76
4.3 Cell Disintegration of MRU5 81
4.3.1 Cell Disintegration Methods 81
4.3.2 Index of Cell Disintegration Methods 98

5 CONCLUSION AND SUGGESTIONS FOR FUTURE WORK 103

REFERENCES 106
APPENDICES 118
BIODATA OF THE AUTHOR 132

LIST OF TABLES

Table		Page
1	The composition of formulated enrichment medium.	38
2	The trace elements composition.	39
3	The vitamin solution composition.	40
4	The concentrations of antibiotics used in this study.	46
5	Result of fermentation reaction of MRU5 on API 20A Kit.	60
6	Growth profile of MRU5 on different antibiotics at various concentrations.	64
7	The growth parameters of MRU5 at different temperatures.	72
8	Maximum extracellular enzymes activity isolated during 14 days of incubation.	78
9	The effect of two physical parameters on the cell disruption of MRU5 by five methods of disruption.	100

LIST OF FIGURES

Figure		Page
1	Phylogenetic tree shows Bacteria and Archaea subdivided into eukaryochaeota and crenarchaeota.	5
2	Flow diagram for preparation and inoculation of media using serum bottle modification of the Hungate technique.	41
3	Effect of temperature on growth of MRU5.	68
4	Effect of pH on growth of MRU5.	69
5	Effect of salinity on growth of MRU5.	70
6	The experimental data of growth of MRU5 cultivated at different temperatures to calculated data according to Gompertz model.	74
7	The effect of temperature on maximum growth rates estimated from modified Gompertz model.	75
8	Enzyme activity and protein concentration of extracellular α-amylase.	76
9	Enzyme activity and protein concentration of extracellular lipase.	77
10	Enzyme activity and protein concentration of extracellular xylanase.	77
11	Total intracellular α-amylase activity.	81
12	The release of α-amylase activity and protein concentration after disruption in bead miller with 3g of glass beads.	84
13	The release of α-amylase activity and protein concentration after disruption in a bead miller with 6g of glass beads.	87
14	The release of α-amylase activity and protein concentration after disruption using lysozyme.	91
15	The release of α-amylase and protein concentration after combination treatment using bead miller (0, 1, 2, 3 and 4 hours) and 0.5 hour homogenizer 8000 rotation per minute.	94
16	The release of α-amylase and protein concentration after combination treatment using bead miller (0, 1, 2, 3 and 4 hours) and 10 min ultrasonication 15kHz.	96
17	α-Amylase enzyme activities after various treatments.	98
18	α-Amylase specific enzyme activity after various treatments.	99

LIST OF PLATES

Plate		Page
1	Gram staining of MRU5 under light microscope (a) at 400X and (b) at 1000X.	59
2	MRU5 under electron microscope (2,500X).	59
3	The sugar fermentation reaction of MRU5 on API 20A Kit.	60
4	Control.	65
5	Growth of MRU5 in media with 25 mg/mL and 50 mg/mL Ampicillin.	65
6	Growth of MRU5 in media with 25 mg/mL and 50 mg/mL Chloramphenicol.	65
7	Growth of MRU5 in media with 30 mg/mL and 50 mg/mL Kanamycin.	65
8	Growth of MRU5 in media with 50 mg/mL Rifampin.	66
9	Growth of MRU5 in media with 200 mg/mL and 400 mg/mL Streptomycin.	66
10	Growth of MRU5 in media with 12.5 mg/mL and 20 mg/mL Tetracycline.	66
11	Growth of MRU5 in media with 200 mg/mL Geneticin.	67
12	Disruption of cell by bead miller treatment with 3g of glass bead after 4 hours.	85
13	Disruption of cell by bead miller treatment with 6g of glass bead at hour 2.	89
14	Disruption of cell by bead miller treatment with 6g of glass bead at hour 3.	89
15	Disruption of cell by using lysozyme at 10 minutes treatment.	92
16	Disruption of cell by using lysozyme at 2 hours treatment.	92
17	Disruption of cell by bead mill treatment after 2 hours followed by homogenization for 0.5 hour.	95
18	Disruption of cell by bead mill treatment after 3 hours followed by sonication for 10 minutes.	97

LIST OF ABBREVIATIONS

C	Carbon
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
eg.	Example
H_2S	Hydrogen sulphite
m	Meter
M	Molar
mg	Milligram
mL	Milliliter
N_2	Nitrogen gas
NaCl	Sodium choride
^oC	Degree Celcius
OD	Optical density
PCR	Polymerase chain reaction
RSS	Residual sum of square
SDS	Sodium dodecyl sulfate
µg	Microgram
µm	Micrometer

CHAPTER 1

INTRODUCTION

Hyperthermophilic and thermophilic bacteria can be isolated from hot springs, hot pipes of factories and other habitats that are only heated transiently and even oil wells. Organisms which growth temperature optimum is above 45^{o}C are called thermophile, while those which optimum is above 80^{o}C are called hyperthermophiles.

Thermostable enzymes can be obtained from mesophilic, thermophilic and hyperthermophilic organisms; even psycrophiles have sonic thermostable enzymes. Hyperthermophiles and thermophiles represent an obvious source of thermostable enzymes, being reasonable to assume that such character will confer their proteins a high thermal stability.

The technological use of thermophiles still faces several challenges since knowledge on physiology and genetics of such organisms is poor, they are fastidious, grow slowly and are not recognized as safe. Therefore, even though thermal stability can be considered a rare event in mesophilic organisms, thermostable enzymes used by industry are still produced from mesophiles and commercial enzymes from thermophiles are still scarce. Opportunities for thermophilic enzymes in industrial processes include in food biotechnology (produce glucose and fructose for sweeteners, baking, and brewing), genetic engineering, paper bleaching, detergents, and bioremediation.

Hyperthermophilic and thermophilic microorganisms offer some major advantages for industrial and biotechnological processes that many run more rapidly and efficiently at high temperatures. Enzymes from these organisms are capable of catalyzing biochemical reactions at high temperatures and are generally more stable than enzymes from mesophiles, thus prolonging the shelf life of enzyme preparations. The enzymes are possible to be utilized in enhanced oil recovery processes, petroleum biodegradation or in degradation of industrial residues and toxic wastes.

Undoubtedly, a microorganism can produce extracellular and/or intracellular or membrane bound enzymes. If the desired enzyme is intracellular or membrane bound, cell disruption is an important early step in product recovery. The methods used for disrupting microbial cells can be classified as mechanical (applying sheer force, agitation with abrasives or freeze thaw), non-mechanical (treatment with acid and alkali, detergents or organic solvents) and enzymatic digestion with lysozyme or proteases.

The physical methods are always the preferred steps for cells disruption at small and large scale. However, the method chosen highly depend on the cells properties, for example the physical strength of the cell wall and the location of the protein interest. More importantly, the method should allow maximum protein released while preserving the protein structure and activity of the protein of interest.

Thus, the objectives of this study were to:

(a) Determine the identity of MRU5,

(b) Determine the growth kinetic of MRU5 including some extracellular thermostable enzyme,

(c) Determine the efficiency of several methods to disintegrate MRU5 cells.

Mathematical models were used to further describe the data obtained.

CHAPTER 2

LITERATURE REVIEW

2.1 Identification of Hyperthermophilic Microorganisms

2.1.1 Taxonomy and Phylogenetic Relation of Hyperthermophiles

During the last decades, a great diversity of hyperthermophilic prokaryotes have been isolated from hydrothermal, geothermal and anthropogenic high-temperature ecosystems (Stetter, 1988 & 1999). By definition, hyperthermophiles grow optimally at temperatures between 80^{o}C and above (Stetter, 1988). In contrast to moderate thermophiles, they are unable to propagate below about 60^{o}C (Stetter, 1999). The highest growth temperature observed is 113^{o}C for *Pyrolobus fumarii* (Biöchl et al., 1997). Due to their metabolic flexibility and their outstanding heat resistance, hyperthermophiles are as interesting for basic research as they are for biotechnological applications (Stetter, 2000).

Figure 2.1 shows that there are about 75 species of hyperthermophilic bacteria and archaea known. Hyperthermophiles are very divergent, both in terms of their phylogeny and phsiological properties, and are grouped into 32 genera in 10 orders (Stetter, 2000). Physiologically, they cover a broad spectrum, ranging from obligate chemolithoautotrophs to strict organotrophs, from aerobes to strict anaerobes or from highly acidophiles to alcaliphiles. Phylogenetically, hyperthermophiles appear in both kingdoms of the domain archaea and in the deep branching phyla of the bacterial domain.

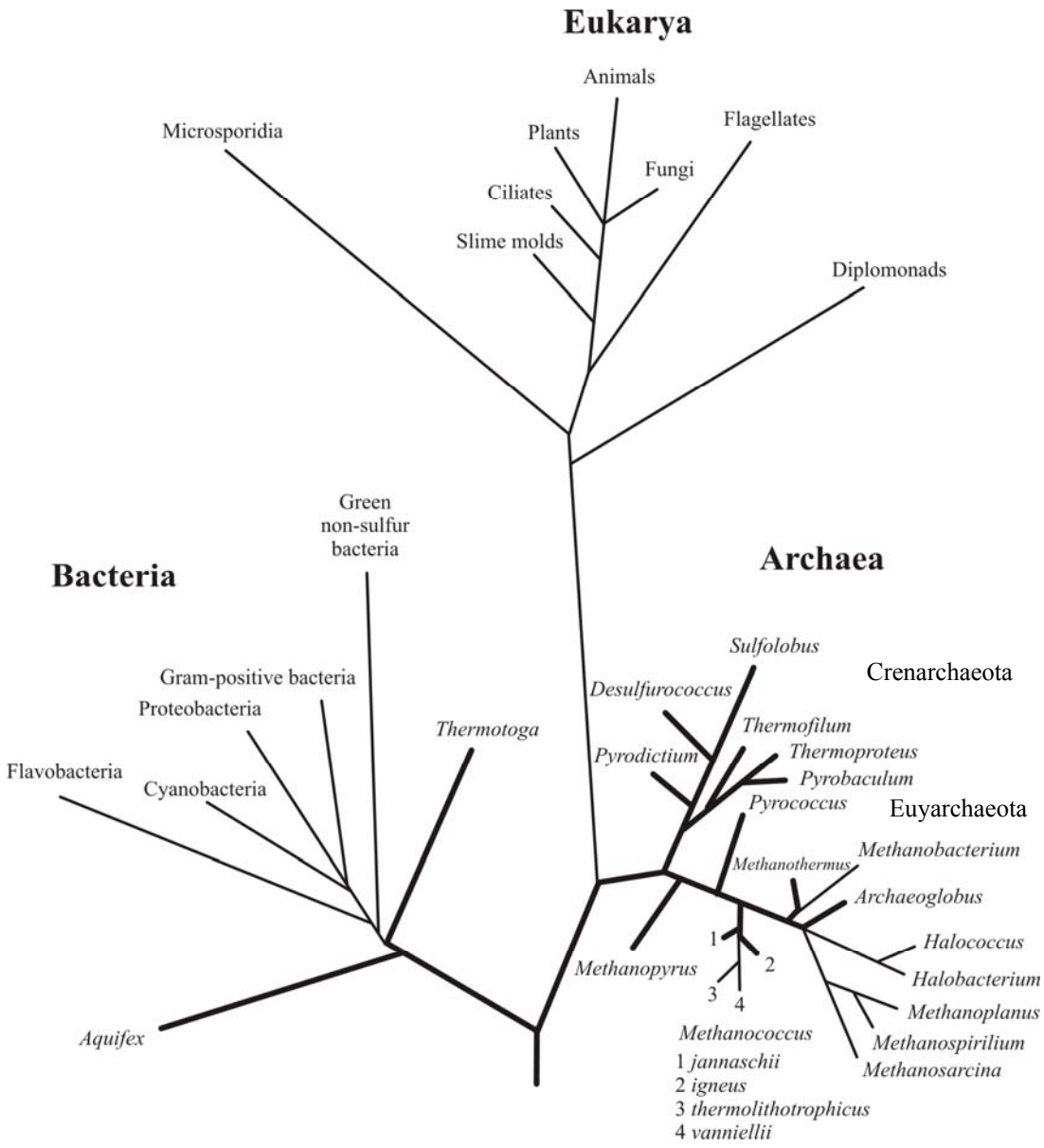

Figure 2.1: Phylogenetic tree shows bacteria and archaea subdivided into eukaryochaeota and crenarchaeota. (Source: Madigan et al., 2002)

Extreme thermophiles, which grow optimally between 60°C and 80°C, are widely distributed among the genera *Bacillus*, *Clostridium, Thermoanaerobacter, Thermus, Fervidobacterium, Thermotoga and Aquifex.* The domain archaea is subdivided into the subdomains Euryarchaeota and Crenarchaeot a.

The subdomain Euryarchaeota consists of methanogens (such as *Methanococcus, Methanobacterium* and *Methanosarcina*), sulfate reducer (*Archaeoglobus*), extreme halophiles (including genera such as *Halobacterium* and *Halococcus*), and extreme acidophilic thermophiles (Schleper et al., 1995). Methanogens grow over the whole temperature spectrum where life is found: from psychrophiles (Nichols and Franzmann, 1992) to mesophiles (Kandler and Hippe, 1977) to extreme thermophiles (Kurr et al., 1991).

Crenarchaeota consists of genera *Sulfolobus, Pyrodictium, Pyrolobus, Pyrobaculum* and *Thermoproteus*.

2.1.2 Methods of Identification

In the recent years molecular phylogenetic analysis has been used to characterize microbial subpopulations and communities in a variety of environments (Amann et al., 1995). Since only 0.001 to 1% of existing bacteria are cultivable (Ward et al. 1990), investigators have turned to modern molecular tools based on the PCR and phylogenetics of the 16S rRNA gene (Woose, 1987). Phylogenetic procedures provide information that may complement or augment the data that is derived from culture-based procedures.

In analyzing environmental samples extraction and purification of DNA can be problematic due to a variety of factors (Lovell and Piceno, 1994). To solve these problems, some investigators have attempted to remove the microbial community from the environmental matrix (Steffan et al., 1988), while others have chosen to

lyse the cells in situ (Tsai and Olson, 1991). The primary concerns of either approach are the efficiency of cell lysis as well as integrity and purity of the extracted DNA. In general the in situ approach produces more quantitative results; the lysis efficiencies can be more than 1 order of magnitude superior compared to cell removal techniques (Lovell and Piceno, 1994). Several investigations have focused on these concerns as they apply to lysis procedures based on bead mill homogenization (Steffan et al., 1988). More et al. (1994) found that a combination of sodium dodecyl sulfate (SDS) and 5 min of bead mill homogenization produced lysis efficiencies of 98% in freshwater sediments. Ogram et al. (1987) found that incubation with SDS at 70°C for 1 hour followed by 5 min of bead mill homogenization produced lysis efficiencies of greater than 90% in marine sediments.

Analysis of rRNA has been widely used to determine the bacterial-species composition of microbial communities. The methodology encompasses such different approaches as direct analysis of rRNA by chromatography and membrane blotting (Stahl et al., 1985). PCR amplification and subsequent cloning and sequencing of reverse-transcribed environmental rRNA (Ward et al., 1990), and denaturing gradient gel electrophoresis (DGGE) of PCR products obtained from extracted bacterial DNA and rRNA (Muyzer et al., 1993).

Biochemical identification tests such as API Campy has been used to identify Thermophilic Campylobacters (Huysmans et al., 1995). API Campy is a miniaturized identification system that uses 11 enzymatic and conventional test plus 9 assimilation and inhibition tests.

2.1.3 Archaea

The distinction between the domains bacteria and archaea is based mainly on the different types of ribosomal RNA and the chemical nature of the membrane lipids: diacyl D-glycerol diester in bacteria versus isoprenoids L-glycerol diethers or di L-glycerol tetraethers in archaea (Woese et al., 1990). In addition, all archaea lack murein, a peptidoglycan with numerous chemical variations that forms rigid cell wall sacculli in almost all taxa of bacteria with only a few exceptions, such as *Mycoplasma, Planctomyces* and *Chlamydia* (Schleifer and Kandler, 1972).

Archaea are found at the limits of the extreme environments described. The reason for this could be contained in the unique structure of the archaeal membrane lipids, which have some features distinct from those of bacterial and eukaryal membranes. The majority of the lipid acyl chains are fully saturated isoprenoids that possess ether links with glycerol. Halobacterial lipids consist of a C_{20}, diether lipid core (Kates, 1996).

In the absence of murein, polymers of diverse chemical natures are found to form rigid cell wall sacculli in the Gram-positive archaea. The majority of the archaea are Gram-negative and possess only proteinaceous or glycoproteinaceous cell envelopes (S-layers), or a reinforcement of the cytoplasmic membrane reminiscent of the glycocalyx of eukaryotic cells (Kandler and König, 1993).

Polysaccharides secreted from halophilic archaea could find use in oil exploration efforts (Rodriguez-Valera, 1992) while secreted haloarchaeal