

**THE MICROBIAL COMPOSITION OF A NATURAL
METHANOGENIC CONSORTIUM**

Nthabiseng Mashaphu



**A thesis submitted in fulfillment of the requirements for the degree of Magister
Scientiae (M.Sc.) in the Department of Biotechnology, University of the Western
Cape.**

Supervisor: Prof. D.A. Cowan

November 2005

Abstract

Wetlands account for approximately 20% of annual global methane emissions. Many wetlands receive inputs of organic matter, nutrients, metals and various toxic compounds from adjacent agricultural and industrial areas. The present study aimed to investigate the microbial composition of a natural methanogenic consortium. A consortium-based molecular approach to study diversity of methanogenic microbial communities in a natural wetland at the primary inflow was used. Key microorganisms of a methane producing consortium were identified. Extracted high molecular mass DNA was analysed by PCR combined with denaturing gradient gel electrophoresis and subsequent sequencing of 16S rDNA. The diversity of methanogenic microorganisms within the consortium was also determined using denaturing gradient gel electrophoresis (DGGE). Analysis showed that methanogens from the wetland were more in species of hydrogenotrophs than acetotrophs, suggesting that the wetland soil contain high acetate concentration. This study was also aimed to identify syntrophic microorganisms in the wetland system. In order to achieve this goal, primers Syn682F and Syn1196R, specific for the families *Syntrophaceae* and *Syntrophobacteraceae*, were designed. Representatives of twenty nine (29) unique restriction patterns were sequenced for phylogenetic analysis. 20 clones from the constructed 16S rDNA library belonged to δ -*proteobacteria*, and 9 clones belonged to α -*proteobacteria*. The data obtained suggest a well established syntrophic relationship within the wetland.

Declaration

“I declare that, **THE MICROBIAL COMPOSITION OF A NATURAL METHANOGENIC CONSORTIUM** is my own work that has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.”

Nthabiseng Mashaphu



Date.....

Signed:

Acknowledgements

I would like to thank **God** for giving me the strength and courage to complete this project.

I thank my supervisor **Prof. Donald Cowan** and co-supervisor, **Dr. Lukas Rohr** for the support and guidance throughout this project.

I thank **my entire family** for their continuous support and encouragement throughout the years of my studies.

All the members and technical staff of Advanced Research Centre for Applied Microbiology Laboratory, thank you for everything.

Dr. William Stafford, Dr. Estela Stenico, an ex-member of Arcam and **Dr. Ndiko Ludidi** , from the Genetics department of University of Stellenbosch, thank you for your valuable academic input and suggestions in my project.

Tumelo Seameco, thank you for introducing me to **Prof. Cowan**.

I wish to thank **Mintek (Randburg, South Africa)**, especially their representatives **Bob Tait and John Neale** for financial support during my Masters studies.

Abbreviations

A:	Adenine
ARCAM:	Advanced Research Centre for Applied Microbiology
ARDRA:	Amplified rDNA Restriction Analysis
ATP:	Adenosine Triphosphate
BLAST:	Basic Local Alignment Search Tool
bp:	Base pairs
C:	Cytosine
DNA:	Deoxyribonucleic Acid
DGGE:	Denaturing Gradient Gel Electrophoresis
EDTA:	Ethylenediamine Tetraacetic Acid
FTIR:	Fourier Transform Infrared
G:	Guanine
GC:	Gas Chromatography
GC-clamp:	Guanosine-cytosine clamp
GenBank:	Nucleotide sequence database
IPTG:	Isopropyl β -D-thiogalactopyranoside
kb:	Kilobase pairs
LB:	Luria Bertani
LiP:	Lignin Peroxidase
m:	Metre
M:	Molar
mM:	Millimolar

MnP:	Manganese Peroxidase
NCBI:	National Center for Biotechnology Information
NIR:	Near Infrared Reflectance
NMR:	Nuclear Magnetic Resonance
ng:	nanogram
nm:	nanometre
OD:	Optical Density
PCR:	Polymerase Chain Reaction
py-FIMS:	Pyrolysis-Field Ionisation Mass Spectroscopy
py-GC:	Pyrolysis Gas Chromatography
PVPP:	Polyvinylpolypyrrolidone
RDP-II:	Ribosomal Database Project-II
rDNA:	ribosomal DNA
rpm:	revolutions per minute
RNA:	Ribonucleic acid
rRNA:	ribosomal RNA
SDS:	Sodium dodecyl sulphate
sp.:	species (singular)
spp.:	species (plural)
Syn:	Syntrophobacterales
T:	Thymine
TEMED:	Tetramethylethylenediamine
TGGE:	Temperature Gradient Gel Electrophoresis



T_m :	Melting temperature
T-RFLP:	Terminal Restriction Fragment Length Polymorphism
Tris:	2-amino-2 (hydroxymethyl) 1,-3-propanediol
μ l:	Microlitres
μ M:	micromolar
UWC:	University of the Western Cape
v/v:	volume per volume
w/v:	weight per volume
X-Gal:	5-bromo 4-chloro 3-indolyl β -D-galactopyranoside



LIST OF FIGURES

Fig. 1.1. Models of various coal compounds.....	6
Fig. 1.2. Schematic representation showing various trophic groups of microorganism involved in bioconversion of organic matter to CH ₄ production during anaerobic digestion processes.....	13
Fig. 1.3. Metabolism of fatty acids that occur during syntrophic relationships.....	17
Fig. 1.4. Phylogenetic hierarchy of methanogenic archaea.....	19
Fig. 1.5. Structures of coenzymes and electron carriers involved in methanogenesis.....	24
Fig. 1.6. Schematic representation of the pathway of methanogenesis.....	25
Fig. 2.1. Sample site and sampling device.....	30
Fig. 3.1. Agarose gel (0.8%) of total DNA extracted from lower core and upper core soil samples using the modified Miller method.....	44
Fig. 3.2. Agarose gel (0.6%) of total DNA extracted from soil samples using the modified Miller method.....	45
Fig. 3.3. PCR products generated from purified DNA using different amounts of template with E9F and U1510R primers.....	47
Fig. 3.4. PCR products generated from purified DNA from upper core and lower core samples.....	48
Fig. 3.5. Colony PCR using pUCM13F and pUCM13R primers.....	49
Fig. 3.6. ARDRA patterns of 16S rDNA PCR products of methanogenic archaea using pUCM13F and pUCM13R primers.....	51
Fig. 3.7. Phylogenetic tree of partial 16S rRNA gene sequences of methanogenic archaea from wetland soil.....	57

Fig. 3.8. DGGE band patterns of PCR products obtained from wetland soil using M340F-GC and M707R primers.....	59
Fig 3.9. Optimisation of PCR for the amplification of syntrophic 16S rDNA using primers Syn682F and Syn1196R.....	63
Fig. 3.10. Colony PCR using pUCM13F and pUCM13R primers.....	65
Fig. 3.11. ARDRA patterns of 16S rDNA PCR products of <i>Syntrophobacterales</i> using pUCM13F and pUCM13R primers.....	68
Fig. 3.12. Phylogenetic tree of partial 16S rRNA gene sequences from domain <i>Bacteria</i> , constructed with Phylo_win 2.0 using the neighbour-joining algorithm.....	74
Fig. 4.1. Alignment showing mismatches of the <i>Methanococcus jannaschii</i> rDNA with the reverse primer M707R.....	81



LIST OF TABLES

Table 1.1. Chemical composition of low rank coal.....	5
Table 1.2. Coal rank.....	7
Table 1.3. Examples of organisms that solubilise coal.....	11
Table 1.4. Cooperation of communities in degradation of organic matter.....	15
Table 1.5. Main characteristics of methanogenic orders.....	20
Table 1.6. Substrates used by methanogens in the production of CH ₄	21
Table 2.1. Primers used in PCR experiments of this study.....	36
Table 2.2. PCR conditions used in this study.....	37
Table 2.3. DGGE gel constituents.....	42
Table 3.1. Quantification of nucleic acids.....	46
Table 3.2. Colony counts after transformation using pUCM13F and pUCM13R primers.....	49
Table 3.3 a. Summary of ARDRA patterns from 16S rRNA genes of methanogenic archaea using <i>AluI</i> restriction endonuclease digestion.....	52
Table 3.3 b. Summary of ARDRA patterns from 16S rRNA genes of methanogenic archaea using <i>HaeIII</i> restriction endonuclease digestion.....	52
Table 3.3 c. Analysis of ARDRA patterns from 16S rRNA gene of methanogenic archaea using <i>RsaI</i> restriction endonuclease digestion.....	53
Table 3.4. Blast analysis of 16S unique rDNA insert sequences.....	54
Table 3.5. Analysis showing targeted genera using Syn 682F and Syn 1196 R primers.....	61
Table 3.6. Colony counts after transformation using Syn682F and Syn1196R	

primers.....	64
Table 3.7 a. Analysis of ARDRA patterns from 16S rRNA genes using <i>MvaI</i> restriction endonuclease digestion.....	69
Table 3. 7 b. Analysis of ARDRA patterns from bacterial 16S rRNA genes using <i>HpyF10VI</i> restriction endonuclease digestion.....	69
Table 3.7 c. Analysis of ARDRA patterns from 16S rRNA genes using <i>SduI</i> restriction endonuclease digestion.....	70
Table 3.8. Blast analysis of 16S unique rDNA insert sequences.....	73



Table of Contents

Title.....	Page
Abstract	i
Declaration	ii
Acknowledgements	iii
Abbreviations	iv
List of figures	vii
List of tables	ix
Chapter 1: Literature review	1
1.1 Introduction	1
1.2 Coal	2
1.2.1 Structure and composition of coal	2
1.2.2 Analytical techniques	5
1.2.3 Coal rank	7
1.3 Bioconversion of coal	7
1.3.1 Biosolubilisation	7
1.3.2 Mechanisms of biosolubilisation	8
1.3.3 Microbiology of biosolubilisation	10
1.3.4 Products of biosolubilisation	11
1.4 Anaerobic microbial processes	12

1.4.1	General scheme	12
1.5	Microbiology and physiology of the anaerobic coal degradation	14
1.5.1	Steps involved in anaerobic coal degradation	14
1.5.2	Cooperation in methanogenic communities	15
1.5.3	Syntrophism with methanogens	16
1.6	Methanogens as key organisms in anaerobic coal degradation	18
1.6.1	Habitat and Ecology of methanogens	18
1.6.2	Importance of methanogens	18
1.6.3	Taxonomy and Morphology of methanogens	18
1.6.4	Substrates used by methanogens	20
1.7	Metabolic reactions in methanogenesis	22
1.7.1	Metabolic pathway of methanogenesis	22
1.7.2	Unique coenzymes and electron carriers in the methanogenic pathway	22
1.7.3	Methane	23
1.8	Wastewater and Wetland systems	26
1.9	Identification of microorganisms in anaerobic environments	27
1.9.1	Background	27

1.9.2	Molecular approaches to the microbial ecology	28
1.10	Aims	29
Chapter 2: Materials and Methods		30
2.1	Sampling site and soil sampling procedures	30
2.2	Metagenomic DNA extraction	31
2.3	RNase treatment and DNA purification	32
2.4	DNA purification by phenol extraction	33
2.5	Ethanol precipitation	34
2.6	Quantification of DNA	34
2.7	Agarose gel electrophoresis	34
2.8	Identification of δ -Proteobacteria	35
2.8.1	Design of oligonucleotide primers	35
2.9	Polymerase Chain Reaction (PCR)	35
2.9.1	PCR optimisation	35
2.9.2	PCR amplification	36
2.10	Cloning of PCR products in <i>E. coli</i> and transformation using pTZ57R/T vector	38
2.11	Enzymes used in the study	39
2.12	Amplified rDNA Restriction Analysis (ARDRA)	39
2.13	DNA sequence and analysis	40
2.14	Storage of Clones in liquid media	41
2.15	Nucleotide sequence accession numbers	41
2.16	Denaturing Gradient Gel Electrophoresis (DGGE)	42

Chapter 3: Results	43
3.1. DNA isolation from winery wetland soil	43
3.1.1. Comparison of metagenomic DNA extraction procedures	43
3.1.2. Purifications of DNA	44
3.1.3. Quantification of DNA and purity analysis	45
3.1.4. PCR amplification	46
3.2. Methanogenic <i>Archaea</i> in winery wetland soil	47
3.2.1. PCR amplification of 16S rDNA	48
3.2.2. 16S rDNA clone library and Amplified rDNA Restriction Analysis (ARDRA)	48
3.2.3. Methanogenic 16S rDNA sequence analysis	53
3.2.4. Diversity of methanogenic archaea using DGGE analysis	58
3.3. Identification of <i>Syntrophic</i> Bacteria	59
3.3.1. Primer design	59
3.3.2. PCR amplification of 16S rDNA	62
3.3.3. 16S rDNA clone library and Amplified rDNA Restriction Analysis (ARDRA)	64
3.3.4. <i>Syntrophobacterales</i> 16S rDNA sequence analysis	70
Chapter 4: Discussion	76
4.1. Analysis of the methanogenic community in the wetland	76
4.2. Analysis of the syntrophic community in the wetland	81
References	84

Chapter 1: Literature review

1.1 Introduction

Coal is an important global resource as it supplies the world with low-cost energy (Wicks, 2004). Not only does it provide energy, but also it is one of the most abundant fossil fuels (Faison, 1991; Cairncross, 2001) and a major source of CH₄ (Wilkins and George, 2002). For example, two countries that benefit substantially from low-cost coal fired electricity are China and South Africa. Coal is not only used as an energy source, but as a resource for production of chemicals and materials (Ziegler and van Heek, 1998). South Africa is the world's sixth largest coal producer with an average of 224 million tons of marketable coal annually and the world's second largest net exporter, with most of its production exported to Europe (www.bullion.org.za). South Africa's coal deposits cover a large area, stretching from Witbank in the Mpumalanga Province, down to the northwest of KwaZulu-Natal Province (Somerset, 2003). Currently in South Africa, the consumption of coal accounts for 74% of the country's energy usage and continues to play an increasingly important role in fulfilling societies energy needs (Levine *et al.*, 1982; Cairncross, 2001).

Biological conversion of coal to valuable products has increased in the chemical industries since it is cost effective and increases utilisation (Engesser *et al.*, 1994). This provides an exciting prospect for economic exploitation, since the organic material of low-rank coal can potentially be converted to CH₄ and other high energy activities. Since South Africa produces a large amount of coal, there is a need to

employ new technologies that can minimise environmental impacts. Therefore, there is a major interest in the development of a technology to recover CH₄ derived from coal using microorganisms.

Several groups (Barik *et al.*, 1991; Faison 1991; Johnson *et al.*, 1994; Volkwein *et al.*, 1994; Panow *et al.*, 1997) have reported the production of CH₄ using coal as the substrate. A conversion of low-rank coal into clean CH₄ by methanogenic microorganisms would have economic advantages since it would be inexpensive and user-friendly (Crawford and Gupta, 1993).

1.2 Coal

1.2.1 Structure and composition of coal



Although coal has been used for years, its structure is still under discussion (Fakoussa and Hofrichter, 1999). The structure of coal is complex; however the complexity varies with coal rank. Coal is derived from lignin and is a product of plant fossilisation (Engesser *et al.*, 1994; Laborda *et al.*, 1997). Lignin is a structural plant polymer that is abundant in plants and has an aromatic structure that consists of phenyl propane subunits that are linked by C-C or C-O-C bonds (Atlas and Bartha, 1998; Fakoussa and Hofrichter, 1999). Most low-rank coals such as lignites and sub-bituminous coal resemble lignin in structure and composition (Fakoussa and Hofrichter, 1999). The structure of coal has an influence on its performance in coal conversion processes such as combustion and gasification (Lu *et al.*, 2001). The

formation of coal starts with the decay of plant material, which is then transformed into low-rank coals (lignite and sub-bituminous coal).

Low-rank coals are relatively rich in moisture, volatile matter, ash and organic components (Faison, 1991; Vassilev *et al.*, 1996). As low-rank coal matures, the organic component (such as aromatic content) increases and moisture is lost in the process (Faison, 1993). This maturation results in a higher rank; i.e., bituminous and anthracite. Within coal substances, there are few hydrolytic bonds but there are interconnected networks of hydroaromatics and cycloalkanes (Catcheside and Ralph, 1999; Budwill, 2003). The chemical linkages within coals such as ethers and C-C bonds are not easily hydrolysed by anaerobic bacteria (Budwill, 2003).



As low-rank coals resemble lignin in complexity, they also consist of compound classes such as hydrophobic bitumen, alkali-soluble humic and fulvic acids and other insoluble matrices (Fakoussa and Hofrichter, 1999). Low-rank coals such as lignite are based on benzene and naphthalene ring structures and sub-bituminous coals on naphthalene (Faison, 1991). Van Krevelen (1993) suggested that the transition from low to high-rank coal is accompanied by a corresponding loss of aliphatic structures and an increase in aromatic ring structures. Lignite contains more three-ring and fewer four-ring structures than does sub-bituminous coal (Faison, 1991).

Low-rank coals have a high content of oxygen incorporated within the organic matrix. The majority of organic oxygen within lignite coals is within ether or

hydroxyl functionalities and the remainder is in ester, carboxyl and carbonyl groups (Faison, 1991). The carboxyl groups that are bound to polyaromatics are dominant in low-rank coals (Sugamo *et al.*, 2003).

Coal is mostly dominated by organic material (Table 1.1), with inorganic material representing only 5 % (Faison, 1991). Apart from carbon and oxygen, organic elements such as sulfur, nitrogen and hydrogen only comprise a minor percentage of coal (Levine *et al.*, 1982). The moisture within the coal structure is introduced from biological material during coalification or as a result of water intrusion from the environment (Faison, 1993)

Macerals in coal

The organic material in coal is enclosed within non-crystalline bodies called macerals (Engesser *et al.*, 1994; Faison, 1991). There are three common types of macerals, each described as having different organic origins; vitrinite, liptinite and inertinite (Faison, 1993). The chemical structure of macerals has been described as a macromolecular assemblage that consists of ring systems (van Heek, 2000). Vitrinite originates from lignified plant tissue and has a shiny appearance in coal. Liptinite originates from fungal and plant spores, waxes and resinous materials, and imparts a dark grayish appearance to coal. Inertinite appears as a white material, and originates from carbonised wood (Faison, 1993).

1.2.2 Analytical techniques

Several technological advances have allowed the characterisation of complex heterogenous substances such as soil organic matter (Evans *et al.*, 2001). These techniques have provided insights into the processes of decomposition and humification. Moreover, analytical techniques may be used to determine the ability of chemical and physical approaches to fractionate carbon into meaningful pools (Evans *et al.*, 2001). Jones *et al.* (1999) review some of the different technologies that have been employed to determine coal structure. These include Fourier Transform Infrared (FTIR) spectroscopy (Schulter and Schnitzer, 1993; Silva-Stenico *et al.*, 2004), Near Infrared Reflectance (NIR) spectroscopy X-ray diffraction, Nuclear Magnetic Resonance (NMR), Pyrolysis-field Ionisation Mass spectroscopy (py-FIMS), pyrolysis gas chromatography (py-GC) and solvent swelling and extraction. Models of coal compounds (Fig. 1.1) have been proposed to describe the molecular structures of various coal ranks (Schulter and Schnitzer, 1993; Silva-Stenico *et al.*, 2004).

Table 1.1. Chemical composition of a low-rank coal (adapted from Levine *et al.*, 1982).

Compound	Content % (wt/wt)
Organic	95
C	73
H	5.2
O	20
N	1
S	< 1
Inorganic*	5

*Includes Si, Al, Fe, Ca and Na.

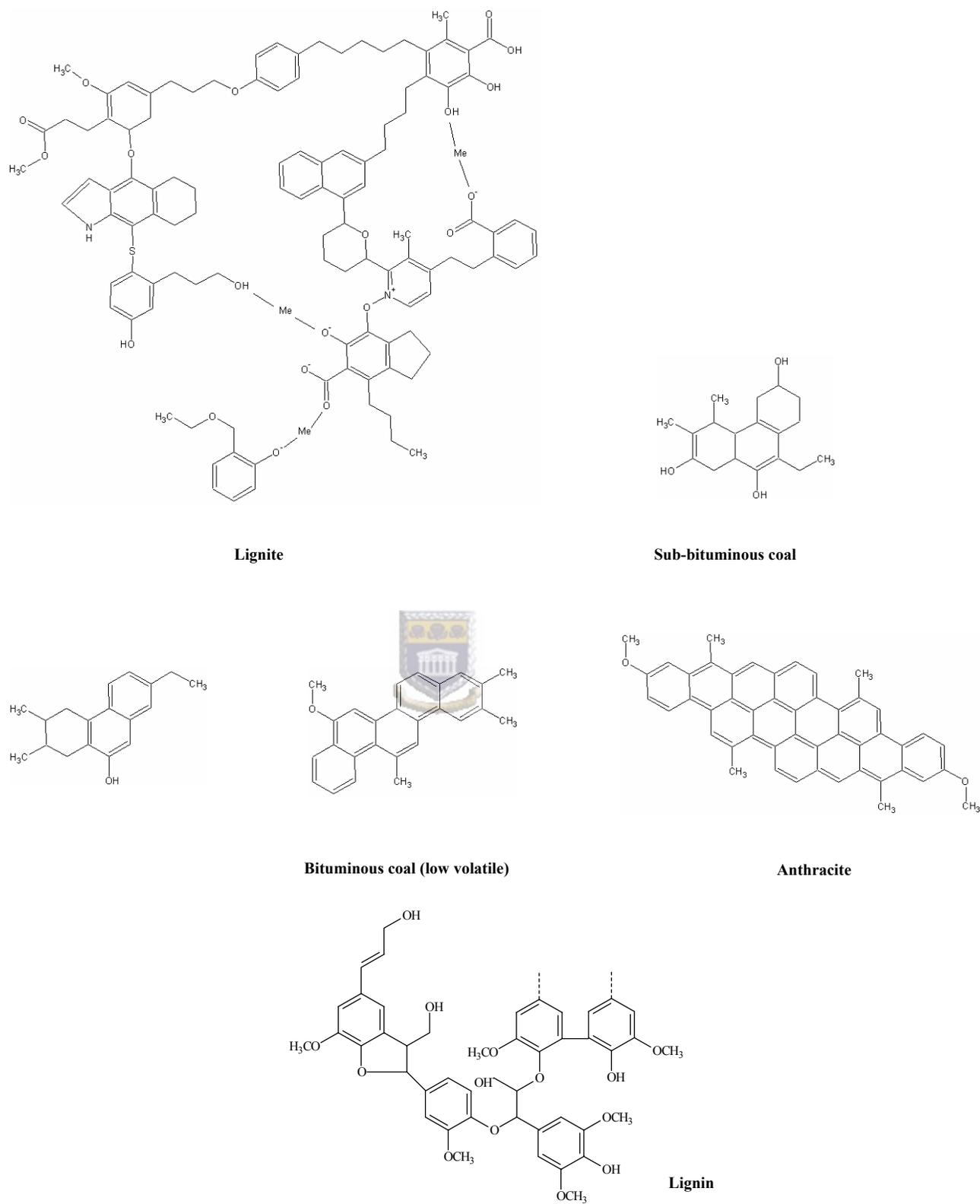


Fig. 1.1. Models of various coal compounds.

1.2.3 Coal rank

Parameters used to classify coals are composition, grade and rank. Rank is defined as the level of coalification of the organic matter and grade refers to the amount of inorganic matter (Polat *et al.*, 2003). The rank of coal is estimated by measuring the moisture content, specific energy, reflectance of vitrinite and volatile matter. Rank of coals is also classified by age and is represented in the Table 1.2.

Table 1.2. Coal rank (adapted from Faúndez *et al.*, 2005).

		Type of coal	Rank stage
		Peat	
Rank Lowest ↓ age ↓ Highest	Brown Coal		Lignite
			Sub-bituminous
	Bituminous		High volatile
			Medium volatile
			Low volatile
			Anthracite and semi anthracite

1.3 Bioconversion of coal

1.3.1 Biosolubilisation

Low-rank coals (lignite and leonardite) are considered as targets for bioconversion technology since (a) they can be converted to fuels because of their higher oxygen content and (b) they have been shown to be significantly more soluble than high-ranks (Ralph and Catcheside, 1997; Larborda *et al.*, 1997; Gockay *et al.*, 2001). The

age of the coal also determines its suitability for biosolubilisation (Faison, 1993) and the susceptibility of low-rank coal is generally parallel to the degree of weathering (Faison, 1991; Catcheside and Ralph, 1999). Most studies on biosolubilisation rely on the pretreatment of coal with oxidising agents such as H_2O_2 , HNO_3 and O_3 which increase the oxygen content (Faison, 1993).

1.3.2 Mechanisms of biosolubilisation

The mechanisms involved in the biosolubilisation process are both enzymatic and non-enzymatic. Mechanisms involved in solubilisation are further classified into a system that can be abbreviated as ABCD (Fakoussa and Hofrichter, 1999). A= alkaline substances, B=biocatalysts, C= chelators, and D=detergents. Most studies have focused more on liquefaction of low-rank coal and desulphurisation of low and high-rank coal (Panow *et al.*, 1997; Laborda *et al.*, 1997). Three mechanisms have been proposed for biosolubilisation of low-rank coal such as lignite: (1) enzymatic attack, (2) non-enzymatic processes such as basic metabolites and (3) microbial chelators (Panow *et al.*, 1997). The simplest method for achieving coal solubilisation is through the production of a surfactant where polar material would be brought into aqueous solution without breaking the covalent bonds (Faison, 1991).

The enzymatic component involves oxidative enzymes that depolymerise and thereby solubilise coal macromolecules. The catabolism of organic macromolecules within low-rank coal requires a number of enzymes that react specifically with one of the many types of substructures in coal (Catcheside and Ralph, 1999).

Approaches that have been used to find enzymes with the ability to catabolise coal are enrichment cultures for microorganisms that can use coal as their sole carbon source and screening of non-specific peroxidases such as those that are synthesised by wood-rot fungi (Catchside and Ralph, 1999). The most important enzymes of the peroxidase group are lignin peroxidase (LiP) and manganese peroxidase (MnP). These enzymes have been identified in coal solubilising fungi such as *Phanerochaete chrysosporium* (Catchside and Ralph, 1999). Ralph and Catchside (1999) suggest that LiP and MnP operate synergistically to convert low-rank coal to products that can be further metabolised by *P. chrysosporium*. LiP is specific for aromatic compounds and can oxidise phenolic and non-phenolic structures. MnP uses Mn (II) and Mn (III) as cofactors (Catchside and Ralph, 1999) and MnP has been used in decolorisation and depolymerisation studies, due to the fact that it can convert high molar mass humic acid to lower molar mass substances. Laccases are lignolytic fungal enzymes that catalyse the oxidation of a range of phenolic substrates while reducing H_2O_2 to H_2O .

The non-enzymatic process for solubilisation of low-rank coal involves alkaline agents and chelators (Crawford and Gupta, 1993). The mechanism of action of basic metabolites has been investigated by Quigley *et al.* (1989 a; b) who showed that a wide range of organisms growing on rich media generate alkaline metabolic products that lead to ionisation of acidic groups in low-rank coals (Catchside and Ralph, 1999).

1.3.3 Microbiology of biosolubilisation

Faison (1991) reports that the presence of microbes in coal was discovered in the early 1960s. The ability of microorganisms to degrade coal was firstly investigated by Rene Fakoussa in Germany in 1981, focusing on how bacteria utilise and solubilise the organic phase of hard coal (Holker *et al.*, 1999; Catcheside and Ralph, 1999). Subsequently, it was shown that wood-rot fungi could quantitatively solubilise the low-rank coal leonardite (Holker *et al.*, 1999; Catcheside and Ralph, 1999). Numerous studies on coal biosolubilisation have since been performed (Laborda *et al.*, 1997; Fakoussa and Hofrichter, 1999).

Since the 1960s, several species of fungi and bacteria have been shown to grow on or transform solid coal (Faison, 1991). Two fungi, *Polyporus versicolor* and *Poria monticola*, were reported to be able to solubilise lignite by Cohen and Gabriele (1982). These two fungi were able to grow directly on crushed lignite particles and on a minimal agar medium supplemented with lignite as a sole carbon source. The result was a dark, water soluble, liquid transformation product (Faison, 1991).

Members of the *Basidiomycetes* are known to be lignin degraders. These organisms produce oxidative enzymes which are thought to be implicated in the mechanism of biosolubilisation (Fakoussa and Hofrichter, 1999). Numerous reports have since demonstrated the biosolubilisation of low and high-rank coals, mostly involving members of the kingdom *Fungi* (Table 1.3).

Table 1.3 Examples of organisms that solubilise coal (adapted from Faison, 1991; Laborda *et al.*, 1997; Holker *et al.*, 1999).

Kingdom	Class	Type of coal	
Fungi	Basidiomycetes <i>Coriolus versicolor</i> <i>Phanerochaete chrysosporium</i> <i>Poria placenta</i> <i>Piptoporus betulinus</i> <i>Coprinus sclerotigenis</i>	Low and High-rank coal Mostly lignite, brown coal, leornadite and bituminous coal	
	Deuteromycetes <i>Trichoderma atroviride</i> <i>Fusarium oxysporum</i> <i>Aspergillus</i> sp.	Low-rank coal Mostly lignite, brown coal and sub-bituminous coal	
	Saccharomycetes <i>Candida</i> sp.	Low-rank coal Lignite and sub-bituminous coal	
	Bacteria	Actinomycetales <i>Streptomyces badius</i> <i>Streptomyces setonii</i>	Low-rank coal Lignite and sub-bituminous coal
		Firmicutes <i>Bacillus</i> sp. Proteobacteria <i>Pseudomonas</i> sp.	Low-rank coal Lignite and sub-bituminous coal

1.3.4 Products of biosolubilisation

The products of coal biosolubilisation are polycondensed aromatic substances that have a high oxygen content relative to the coal substrate (Faison, 1991). Possible products of biosolubilisation of low-rank coal include aromatic structures such as benzene, phenols, naphthalenes and anthracenes as well as heterocyclic and aliphatic

compounds (Faison, 1993). Davison *et al.* (1990) demonstrated that the products of coal biosolubilisation were of high molar mass. Both aliphatic bridges and cyclic structures are considered to be targets for enzymatic attack by ligninolytic enzymes.

The modification of powdered hard coal (Hofrichter *et al.*, 1997) by the fungus *Coprinus sclerotigenis* C142-1 was shown to liberate 2-hydroxybiphenyl, alkylated benzenes and polycyclic aromatic hydrocarbons (PAH). Conversely, the liquid coal fraction generated by *Lentinula edodes* yielded fatty acid esters, methyl ethoxybenzenes, ethyl methoxybenzenes, phenol and N-ethylsuccinimide, together with diethyl esters of humic acids and azelaic acid, the latter being compounds not found in the original humic acid fraction (Götz and Fakoussa, 1999).



1.4 Anaerobic microbial processes

1.4.1 General scheme

Anaerobic digestion is one of the main processes used for sludge stabilisation but is further used for treatment of manure, industrial wastewaters and the organic fraction of municipal solid waste (Gavala *et al.*, 2003). In this biochemical degradation, complex organic molecules such as carbohydrates are broken down in the absence of oxygen into simpler substances such as ethanol, CO₂ and H₂O by facultative or obligate anaerobes (Berry *et al.*, 1987; Ahring, 2003). A major value of this digestion is linked to the production of biogases, CH₄ and CO₂ (Fig. 1.2). The main advantages of anaerobic digestion are that small amounts of biomass are produced, requiring less

nutrients and energy than an aerobic treatment process whereas the generated biogas can be used as an energy source (Gavala *et al.*, 2003).

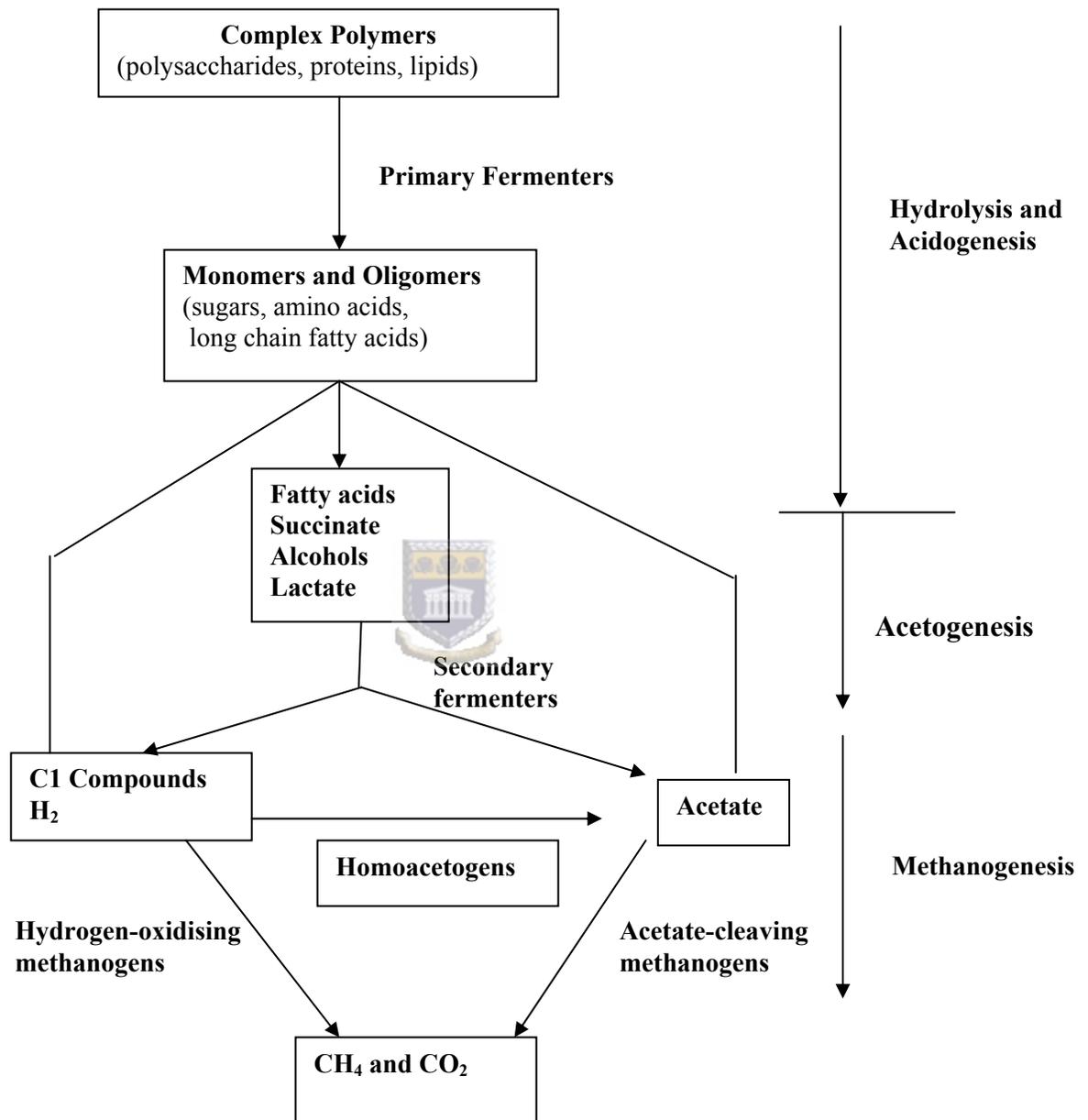


Fig. 1.2. Schematic representation showing various trophic groups of microorganisms involved in bioconversion of organic matter to CH₄ production during anaerobic digestion processes (adapted from Schink, 1997; Conrad, 1999; Ahring, 2003).

1.5 Microbiology and physiology of the anaerobic coal degradation

1.5.1 Steps involved in anaerobic coal degradation

The microbiology of anaerobic digestion is complicated since it involves several bacterial groups, each performing a separate part of the overall degradation process (Gavala *et al.* 2003). Fig. 1.2 illustrates the four steps that are involved in anaerobic digestion. The environment of this digestion accommodates a variety of microorganisms such as acidogens, acetogens and methanogens (Fig. 1.2) which will produce CO₂ and CH₄ as the main products (Madigan *et al.*, 2000; Gavala *et al.*, 2003).

Hydrolysis of macromolecular structures is usually carried out by extracellular hydrolases (hydrolases) which may or may not be the rate-limiting step of the bioconversion process under anaerobic conditions. The rate of the hydrolysis process is dependant on parameters such as particle size, pH, production of enzymes, diffusion and adsorption of enzymes to particles (Gavala *et al.*, 2003). Fermentative bacteria excrete enzymes that hydrolyse organic polymers and catabolise monomers to alcohol, fatty acids, H₂ (Conrad, 1999). During protein hydrolysis, amino acids and peptides serve as energy substrates for anaerobic microorganisms (Stams *et al.*, 2003). Acidogenesis involves the biodegradation of organic matter to volatile fatty acids and alcohols by a heterogenous population. Acetogenesis is classified into two types, acetogenic hydrogenations and dehydrogenations. Acetogenic hydrogenations include the production of CH₃COOH as sole end product either from fermentation of hexoses or from CO₂ and H₂. This step is usually involved in anaerobic digestion

referred to as acetogenic dehydrogenation and in specific to the anaerobic oxidation of long and short volatile fatty acids (Gavala *et al.*, 2003).

1.5.2 Cooperation in methanogenic communities

The conversion of complex organic matter to CH₄ and CO₂ in a natural habitat is possible by the actions of at least four different groups of microorganisms (Figure 1.2 and Table 1.4); primary fermenting bacteria, secondary bacteria (syntrophic bacteria or acetogenic bacteria) and two types of methanogenic *Archaea* (Schink, 1997; Ahring, 2003; Kates *et al.*, 1993 and Gavala *et al.*, 2003). These microorganisms occur naturally and have different roles in the overall process for anaerobic digestion process (Schink, 1997; Conrad, 1999; Ahring, 2003).

Table 1.4. Cooperation of communities in degradation of organic matter (adapted from Nealson, 1998; Valdez-Vazquez *et al.*, 2004).

Microorganisms	Electron donor	Electron acceptor	Product	Type of reaction
Fermenters	Organic C	Organic C	CO ₂	Fermentation
Syntrophs	Organic C	Organic C	H ₂	Acetogenesis
Acetogens	Organic C/H ₂	CO ₂	CH ₃ COOH	Acetogenesis
Methanogens	Organic C/H ₂	CO ₂	CH ₄	Methanogenesis

1.5.3 Syntrophism with methanogens

Syntrophism is a special case of symbiotic cooperation between two metabolically different types of bacteria which depend on each other for degradation of certain substrates (Schink, 1997). This cooperation allows microbial populations to perform activities such as the synthesis of a product, which neither population could perform on its own (Atlas and Bartha, 1998). *Archaeal* populations that are involved in methane production have synergistic relationships with bacterial and other microbial communities (Figs. 1.2 and 1.3). The names of these bacterial genera indicate their syntrophic relationships with hydrogen-consuming methanogenic *Archaea* (Atlas and Bartha, 1998).

Syntrophic or acetogenic bacteria further degrade alcohols and fatty acids to CH_3COOH , H_2 (alternatively formate) and CO_2 (Conrad, 1999; Ahring, 2003). The cooperation is feasible only when hydrogen is at low concentration, so that bacteria of the genera *Syntrophobacter*, *Syntrophomonas* and *Syntrophus* must live in coculture with hydrogen utilising bacteria such as methanogens, acetogens, and sulfur reducers (Nealson, 1997). *Syntrophomonas* species oxidise butyric acid and caproic acid to CH_3COOH and H_2 .

Members of this genus also oxidise valeric acid and enanthic acid to CH_3COOH , CO_2 , and H_2 . Propionate oxidation is an important step in the methanogenesis process, which requires obligately syntrophic consortia of acetogenic bacteria and methanogenic *Archaea* (de Bok *et al.*, 2005). Most of the syntrophic propionate-oxidising bacteria isolated so far belong to the *Syntrophobacter* cluster within the

'*Delta-proteobacteria*'. The CH_3COOH and H_2 produced by these bacteria are used by methanogenic *Archaea* in order to produce CH_4 (Atlas and Bartha, 1998). *Syntrophobacter* species are able to use sulfate as the electron acceptor for propionate oxidation.

Syntrophic propionate oxidation is not restricted to Gram-negative bacteria. Wu *et al.* (1992) described a Gram-positive, spore forming syntrophic propionate-oxidising bacterium (de Bok *et al.*, 2005). Two thermophilic, Gram-positive, syntrophic propionate-oxidising bacteria have been described, *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* TPO^T, which grow axenically on several substrates and, like *Syntrophobacter* strains, can use sulfate as an electron acceptor (de Bok *et al.*, 2005). Under certain conditions, *Desulfovibrio* can supply *Methanobacterium* with CH_3COOH and H_2 from anaerobic respiration and fermentation, using sulfate and lactate to generate these products. *Methanobacterium* can then use the products from *Desulfovibrio* to reduce CO_2 to CH_4 (Atlas and Bartha, 1998).

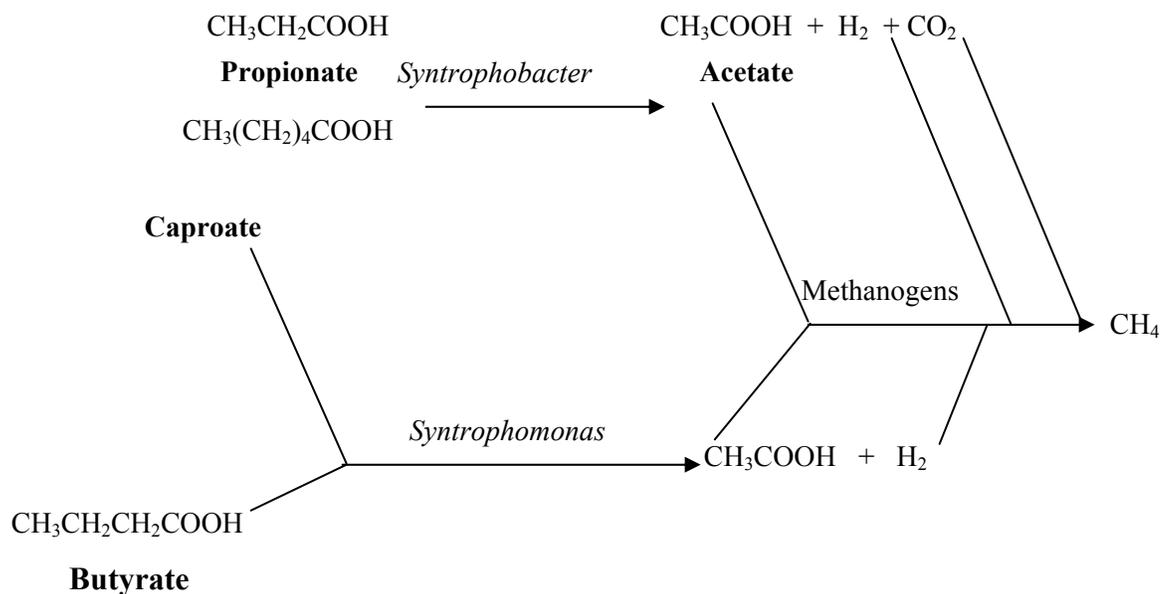


Fig. 1.3 Metabolism of fatty acids that occur during syntrophic relationships (Atlas and Bartha, 1998).

1.6 Methanogens as key organisms in anaerobic coal degradation

1.6.1 Habitat and Ecology of methanogens

As strictly anaerobic microorganisms, methanogens inhabit anoxic environments such as the guts of ruminants and animal digestive tracts, undersea hydrothermal vents and swamps, marshes, paddy fields, sewage sludge and moist landfills, and as endosymbionts of certain protozoa (Kates *et al.*, 1993; Yanga *et al.*, 2004). Methanogens are extremely sensitive to temperature and pH fluctuations and are inhibited by high levels of volatile fatty acids and substrates (Gavala *et al.*, 2003). Both thermophilic and mesophilic methanogens have been isolated (Yanga *et al.*, 2004). They are referred to as chemolithotrophic because they are capable of using CO₂ as their only carbon source (Atlas and Bartha, 1998).



1.6.2 Importance of methanogens

Methanogens are very important organisms for a number of reasons, but mostly for the ability to convert organic matter to CH₄ gas (Conway de Macario and Macario, 2003). These organisms have also created an interest in the pharmaceutical industries because they have been reported as potential sources for Vitamin B₁₂ (Yanga *et al.*, 2004).

1.6.3 Taxonomy and Morphology of methanogens

All living cells have been classified into three main lines of evolutionary descent based on comparative analyses of sequences of the small subunit of ribosomal RNA.

The phylogenetic domains are *Archaea* (formerly known as archaeobacteria), *Bacteria* and *Eukarya* (Conway de Macario and Macario, 2003). The *Archaea* are further divided into phyla, with the most prominent being the *Crenarchaeota* and *Euryarchaeota*. Methanogens constitute a major taxonomic and phenotypic group within the *Euryarchaeota* (Fig. 1.4) (Trevan *et al.*, 1987; Conway de Macario, and Macario, 2003; Watanabe *et al.*, 2004).

Methanogens are morphologically very diverse, ranging from 0.4 μm to 1.7 μm in size. They occur as rods, cocci, spirilla, filaments, and plate shapes (Table 1.5) (Karakashev *et al.*, 2005).

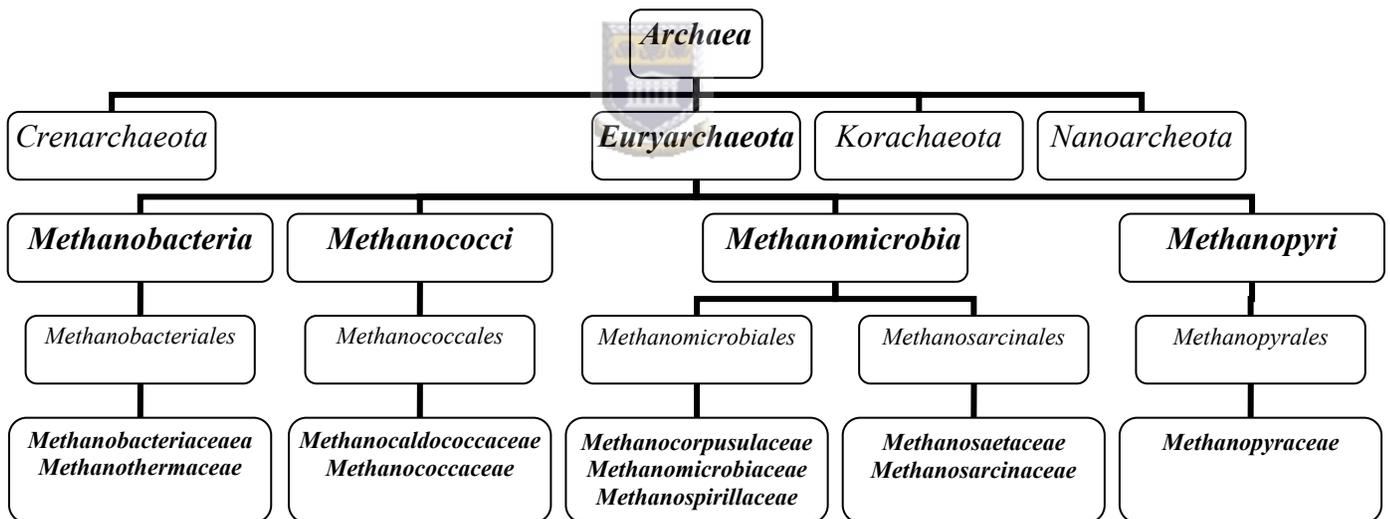


Fig. 1.4. Phylogenetic hierarchy of the methanogenic *archaea*.

Table 1.5. Main characteristics of methanogenic orders (adapted from Karakashev *et al.*, 2005).

Order	Cell morphology	Physiology
<i>Methanobacteriales</i>	Rods or cocci	Hydrogenotrophic mesophilic or thermophilic
<i>Methanococcales</i>	Irregular cocci	Hydrogenotrophic mesophilic or thermophilic
<i>Methanomicrobiales</i>	Small rods, irregular cocci spirilla	Hydrogenotrophic mesophilic or thermophilic
<i>Methanosarcinales</i>	Rods or filaments, irregular cocci or <i>Sarcina</i> -like cells	Strict acetoclastic (<i>Methanosaetaceae</i>), Acetoclastic /hydrogenotrophic (<i>Methanosarcinaceae</i>) Mesophilic or thermophilic

1.6.4 Substrates used by methanogens

Most methanogens utilise a limited number of simple organic substrates, the most important being CH_3COOH and $\text{H}_2\text{-CO}_2$ (Conrad, 1999; Galand *et al.*, 2003). A majority of methanogens are only able to use H_2 and CO_2 for growth (Table 1.6), although some use CH_3COOH , CH_3NH_2 and HCOOH (Kates *et al.*, 1993; Claassen *et al.*, 1999). Methanogens can therefore be divided into two major groups on the basis of the substrates utilised: the hydrogenotrophic methanogens, which use only H_2 and CO_2 as illustrated in reactions 1 and 2 (Conrad, 1999), and those capable of the reduction of methyl groups (illustrated in reaction 3), such as members of the order *Methanosarcinales*, which utilise simple compounds such as acetate as substrates for growth (Vignais *et al.*, 2001). Acetate is an important methanogenic substrate in nature, 70% of methane formed biologically being produced from this acid.



Table 1.6. Substrates used by methanogens in the production of CH₄ (adapted from Kates *et al.*, 1993).

Order	Genus	Major Substrates
Methanococcales	<i>Methanococcus jannaschii</i>	H ₂ + CO ₂ , CH ₃ COOH
	<i>Methanococcus</i>	methanol, methylamine
Methanopyrales	<i>Methanopyrus</i>	H ₂ + CO ₂
Methanomicrobiales	<i>Methanogenium</i>	H ₂ + CO ₂ , formate
	<i>Methanoculleus</i>	H ₂ + CO ₂ , formate, alcohols
	<i>Methanocorpusculum</i>	H ₂ + CO ₂ , alcohols, formate
Methanosarcinales	<i>Methanosarcina</i>	H ₂ + CO ₂ , methanol, methylamines, CH ₃ COOH
	<i>Methanolobus</i>	methanol, methylamines
	<i>Methanotherix</i>	CH ₃ COOH
	<i>Methanohalophilus</i>	methanol, methylamines,
	<i>Methanosaeta</i>	CH ₃ COOH
Methanobacteriales	<i>Methanobacterium</i>	H ₂ + CO ₂ , formate
	<i>Methanosphaera</i>	H ₂ + methanol

1.7 Metabolic reactions in methanogenesis

1.7.1 Metabolic pathway of methanogenesis

The methanogenic pathway where CO_2 is converted to CH_4 involves a variety of specialised coenzymes and enzymes (Fig. 1.5). In the first step, CO_2 is bound to methanofuran (MFR) and is further reduced to the methenyl, methylene, methyl, and finally methane levels, while successively bound to coenzymes tetrahydromethanopterin, 2-methylthioethanesulfonic acid, and 2-mercaptoethanesulfonic acid (Atlas and Bertha, 1998). Hydrogenase is responsible for the assimilation of H_2 using coenzyme F_{420} as an electron acceptor. Since most methanogens use H_2 as a source of electrons, the hydrogenase is found in abundance. The methanopterin is responsible for the stepwise reduction of CO_2 to the methyl group of the CH_3COOH (Atlas and Bertha, 1998; Madigan, 2000; Kates *et al.*, 1983). The methyl group joins the carbonyl group by carbon monoxide dehydrogenase enzyme.

1.7.2 Unique coenzymes and electron carriers in the methanogenic pathway

The methanogenic pathway involves an array of coenzymes with no flavins or quinones, as noted in Fig. 1.5-1.6 (Trevan *et al.*, 1987; Kates *et al.*, 1999; White, 1999; Madigan *et al.*, 2000). The metabolism of methanogens is unique because the pathway requires coenzymes found in no living organisms other than the methanogens (White, 1999; Madigan *et al.*, 2000).

In the metabolic pathway, MFR, methanopterin and coenzyme M are methanogen C1 carriers, while coenzymes F₄₂₀ and coenzyme B are electron donors (Trevan *et al.*, 1987; Kates *et al.*, 1999). 5-deazaflavin (F₄₂₀) is an electron-transferring coenzyme used by several enzymes such as hydrogenase, formate dehydrogenase (Kates *et al.*, 1993; White, 1999), methylene tetrahydromethanopterin (H₄MPT) dehydrogenase, methylene H₄MPT reductase, and heterodisulfide reductase. As mentioned in 1.7.1, MFR participates only in the initial steps of the methanogenic pathway, where CO₂ is bound to the furan at the primary amine. It is then reduced to the formyl level and then transferred to the next coenzyme tetrahydromethanopterin (Kates *et al.*, 1993). H₄MPT participates as four different species and with the carbon in three different oxidation stages.



1.7.3 Methane

Methane gas produced through methanogenesis, is an important potential fuel source for chemical industries. CH₄ can be used in the generation of mechanical, electrical, and heat energy (Pind *et al.*, 2003). It can be used as a fuel source for homes and industry by transmission through natural gas pipelines and converted by microbial action or chemical means to methanol, which can be used as fuel in internal combustion engines (Atlas and Bartha, 1998). Methane is, unfortunately, also a big contributor to global warming.

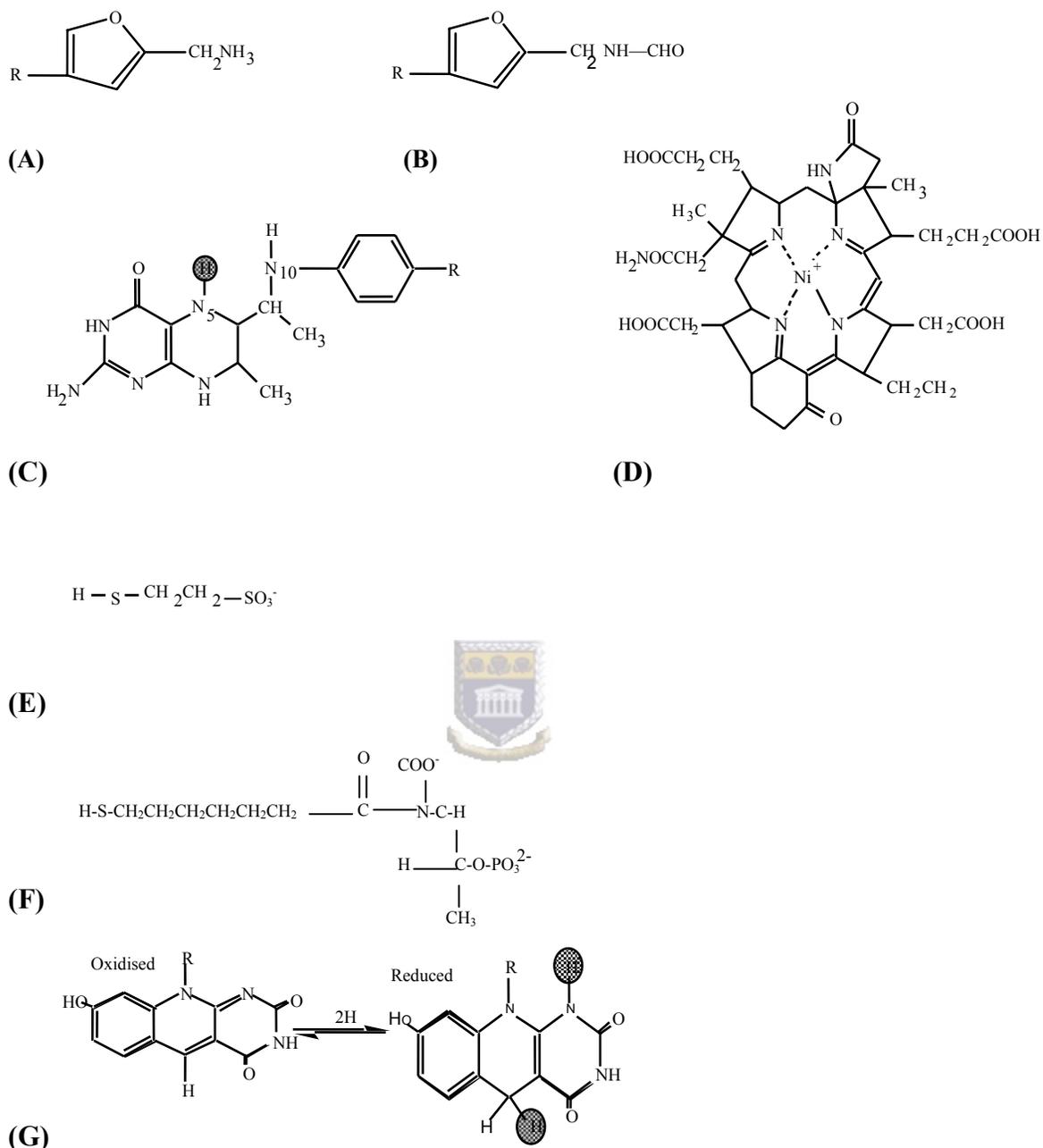


Fig. 1.5 Structures of coenzymes and electron carriers involved in methanogenesis (adapted from Kates *et al.*, 1993). (A), Methanofuran; (B), Formyl-MFR; (C), tetrahydromethanopterin; (D), Coenzyme F₄₃₀; (E), Coenzyme M; (F), N-7-metacaptioheptanoylthreonine phosphate (H-S-HTP), and (G) Coenzyme F₄₂₀.

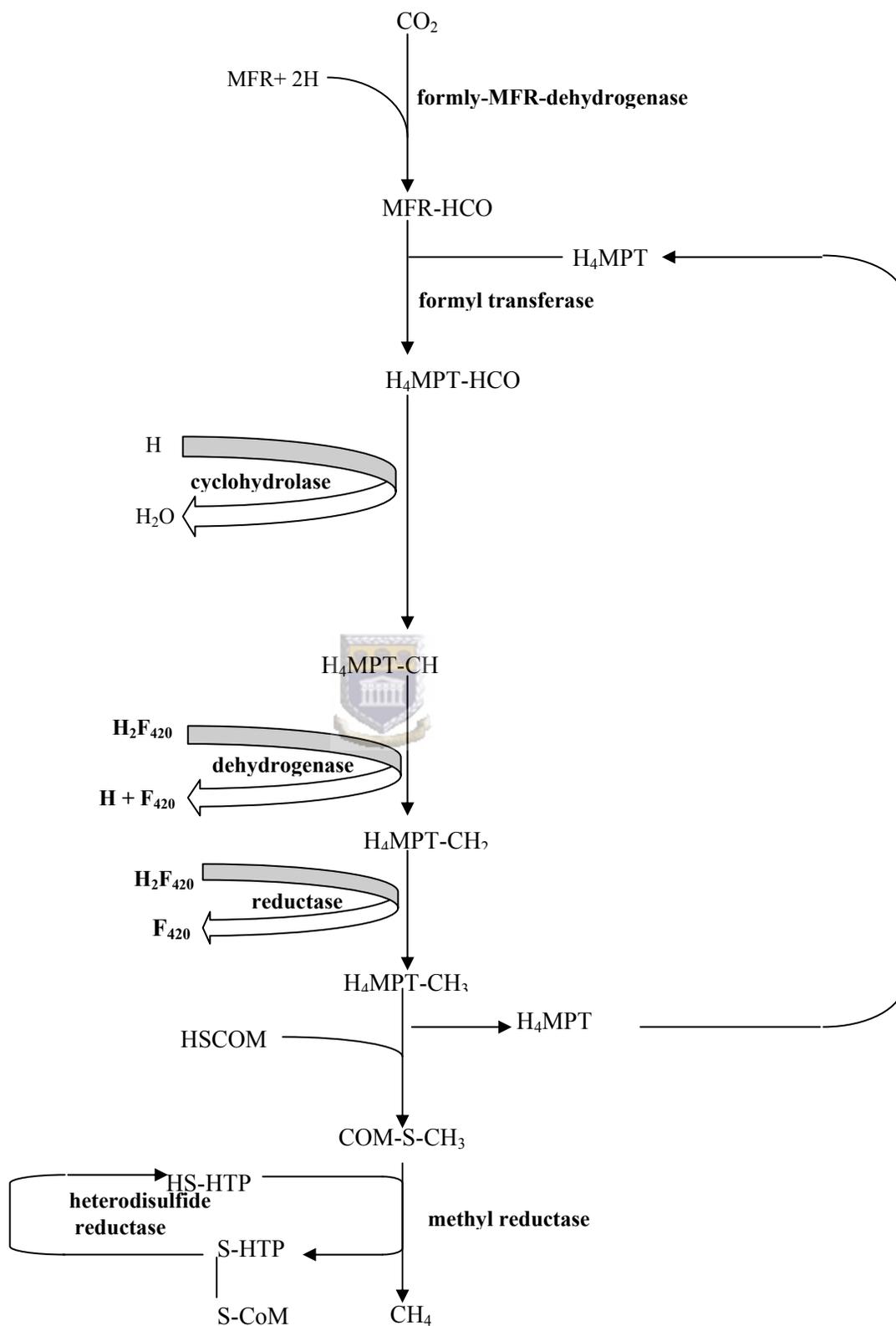


Fig. 1.6 Schematic representation of the pathway of methanogenesis. Intermediates are abbreviated as in the text (adapted from Kates *et al.*, 1993).

1.8 Winery wastewater and wetland systems

The world wine production rate is approximately 2.65×10^8 hl/year of which 63% comes from the European Union (Petruccioli *et al.*, 2000). Wine production is one most highly represented agro-industry in Mediterranean countries since it produces large volume of effluents with organic contents, polyphenol compounds and acidic pH (Eusébio *et al.*, 2005). Industrial wastewaters, for example, breweries and agricultural wastewaters from animal confinements are ideal for processing because they contain high levels of easily degradable organic material (Angenent *et al.*, 2004). Large amount of wastewater originate mainly from washing of equipment and bottles and from cooling (Petruccioli *et al.*, 2000). Composition of winery wastewater have revealed that ethanol, secondary compounds including residual sugars represent more than 90% of organic load of winery effluent (Colin *et al.*, 2005). Winery wastewaters are equated with industrial or urban effluents and as a result, they are treated with the same technologies: natural or forced evaporation, aerobic degradation, active sludge and methanisation (Colin *et al.*, 2005). This proves that it is worthwhile to recover the organic load of winery wastewater rather than dissipating it into sludge and CO₂.

Wetlands account for approximately 20 % of annual global methane emission (Chauhan *et al.*, 2004). Constructed wetlands are used to treat acid drainage from surface or underground coal mines (Nicomrat *et al.*, 2006). A common feature among all constructed wetlands is that they function as a reservoir for secondary minerals that precipitate out of the receiving waters. However little is known about the microbial communities in the receiving wetland cells (Nicomrat *et al.*, 2006).

1.9 Identification of microorganisms in anaerobic environments

The study of microbial ecology requires identification of microorganisms based upon a comprehensive classification system that reflects the evolutionary relatedness of microorganisms (Hofman-Bang *et al.*, 2003). In any detailed study of microbial ecology in, the following three major objectives have to be achieved:

- 1) identification and classification of microorganisms,
- 2) quantification of microbial abundance, and
- 3) quantification and identification of activity.

1.9.1 Background

Morphology and other phenotypic traits have traditionally been used for identification and quantification of microbial populations (Hofman-Bang *et al.*, 2003). Grotenhuis *et al.* (1991) microscopically counted cell numbers of methanogens and identified acetoclastic methanogens based on morphology, and hydrogenotrophic methanogens by visualizing autofluorescence at 420 nm. Culturing of methanogens is difficult due to their low growth rates and fastidious nutritional and environmental requirements.

Phylogenetic analysis allows the identification of microorganisms based on a molecular sequence, eliminating the need for cultivation. This implies that nucleic acids sequences can be retrieved from an environmental sample, sequenced, and compared to known sequences for identification of related organisms (Hofman-Bang *et al.*, 2003).

1.9.2 Molecular approaches to microbial ecology

Molecular approaches allow the use of informational molecules like rRNA for the direct characterisation of environmental communities, for inference of the amounts of total and specific microbial biomass, and even location of specific species within a given environment (Nealson, 1997). The retrieval of nucleic acid sequences from environmental samples is used to investigate microbial communities without cultivation. The nucleic acid fraction that is obtained should ideally represent the whole microbial diversity present in the sample.

Typically, after extraction of environmental DNA, rRNA gene fragments are specifically amplified by PCR, after which the products can be cloned or separated by denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) or (T-RFLP) terminal restriction fragment length polymorphism. The rDNA clone library or the PCR products from the electrophoresis can be sequenced and the sequences obtained may be used to generate phylogenetic trees (Hofman-Bang *et al.*, 2003).

1.10 Aims

This study aims to identify the key microorganisms of a methane producing consortium using a molecular approach. This will include techniques such as DNA extraction and cloning of 16S rDNA amplified by PCR. This study aims to confirm that samples collected from anaerobic sediments contain organisms suitable for processing organic compounds such as CH_3COOH in order to produce CH_4 . This objective involves an analysis of phylogenetic diversity of methanogenic microorganisms using denaturing gradient gel electrophoresis (DGGE).



2 Materials and Methods

2.1 Sampling site and soil sampling procedures

The soil samples were collected at the primary inflow of the wetland of the Middelvlei winery near Stellenbosch, SA (Fig. 2.1A). Acrylic corers with an inner diameter of 2.5 cm and a length of 30 cm were used as sampling devices (Fig. 2.1 B). Prior to sample collection, the corers were soaked in 5 % (w/v) sodium hypochlorite for 30 min, rinsed with sterile water, then with 70 % ethanol and left in the fume hood overnight to dry. The soil cores were sectioned into two layers: 0-5 and 5-15 cm, referred to as *upper core* and *lower core* samples, respectively. The samples were transferred to sterile plastic bags, transported on ice and stored within 2 hrs at -80°C.

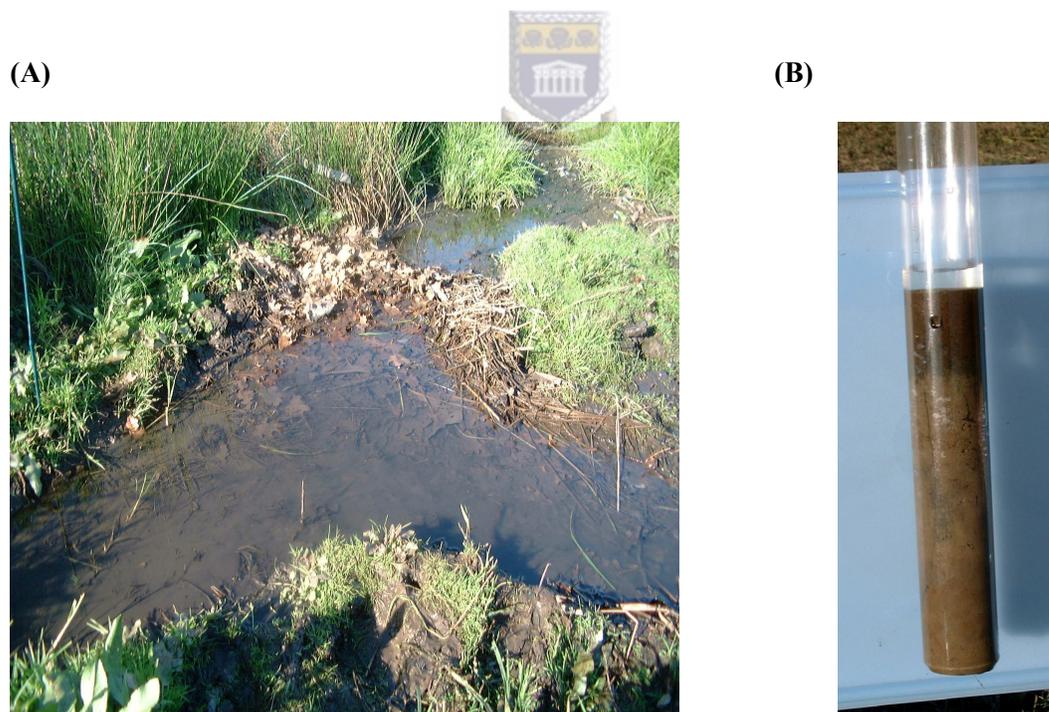


Figure 2.1 Sample site and sampling device. **A**, Primary inflow of the Middelvlei Winery Wetland. **B**, Sediment core taken from inflow area at a depth of 5 cm-15 cm.

The reasons for choosing this location as a sample site were the followings: 1) The winery wastewater flowing through the wetland contains a high quantity of phenolic substances (such as catechol), that are also present in coal. Therefore this soil is likely to contain microorganisms that have the ability to degrade coal compounds. 2) Bubbles at the primary inflow point of the wetland indicate the production of gases, probably CO₂ and methane. This suggests the occurrence of methanogenic microorganisms in this soil and therefore we assume the presence of a microbial consortium that is capable of converting phenolic substances into methane.

2.2 Metagenomic DNA extraction

Three DNA extraction methods were used to determine the most efficient method for wetland soil samples, this included optimising the extraction methods where DNA was extracted from frozen samples and also from freshly collected samples. Metagenomic DNA was extracted directly from 0.5-g aliquots of sediment using the extraction method of Miller *et al.* (1999), with several modifications. Briefly, after thawing on ice, 0.5 g of soil was weighed in a 2 ml screw-capped tube containing 0.5 g of quartz sand (Sigma S-9887). 300 µl each of 100 mM sodium phosphate (pH 8.0) and lysis buffer (100 mM NaCl, 500 mM Tris-Cl [pH 8.0] and 100 g/l SDS) were added and the tube was inverted several times to mix. After the addition of 300 µl of chloroform/isoamyl alcohol (24:1, v/v), the mixture was vortexed for 120 s at maximum speed (Chiltern MT19) and the cell debris were pelleted by centrifugation at 15000 x g for 5 min (Eppendorf). Ammonium acetate (7 M) was added to the supernatant to a final concentration of 2.5 M before the tube was shaken

by hand to mix and centrifuged at 15000 x g for 10 min. The supernatant was transferred to a new microcentrifuge tube and centrifuged at 15000 x g for 3 min. The DNA was precipitated by addition of 0.6 volumes of isopropanol to the supernatant, incubated at room temperature for 15 min and centrifuged at 15000 x g for 10 min. The pellet was desalted with 1 ml of 70% EtOH, air-dried and dissolved in 120 µl of TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0).

Preparation of genomic DNA from *E. coli*

10 µl of liquid culture from ARCAM culture collection (UWC) was inoculated into Luria Bertani medium (10 g bacto[®]-tryptone; 5 g bacto[®]-yeast extract and 5g NaCl) and the culture was incubated overnight at 37°C in a shaker. Following the incubation period, the culture was centrifuged for 10 min at 15000 x g and 10 mg of culture was placed in a 1.5 ml microcentrifuge tube and resuspended in 200 µl of TE buffer. The DNA was purified using the Fermentas Genomic DNA Purification Kit (KO519), according to the manufacturer's instructions.

2.3 RNase treatment and DNA purification

After dissolving the DNA pellet in 120 µl of TE, 0.5 µl of RNase (10 mg/ml, Fermentas) was added in order to remove RNA co-purified during DNA extraction, and the solution was incubated at room temperature for 30-60 min. The DNA was further purified using polyvinylpolypyrrolidone (PVPP, Sigma P-6755) and Sephacryl S-500 HR (Amersham Biosciences 17-0613-10). For this procedure, the caps of 1.5 ml and the lower part of 0.6 ml microcentrifuge tubes were cut, a 20-µl

filter tip was cut 2 mm below the filter. For the construction of the column, a filter tip was placed into a 0.6 ml tube and both the tip and the tube were placed together in a 1.5 ml tube. Self-constructed minicolumns, packed with 400 μ l of a 100 g/l PVPP suspension, were loaded into a 20- μ l pipette tip with filter. The column was washed with 300 μ l of TE, and centrifuged at 200 x g for 2 min and dried by centrifugation at 600 x g for 10 min. The crude DNA was loaded onto the column, and after a 1 min room temperature incubation period was eluted by centrifugation at 600 x g for 5 min and then at 1700 x g for 10 min (Berthelet *et al.*, 1996; Jackson *et al.*, 1997; Sambrook *et al.*, 2001).

For Sephacryl adsorption, the same procedure for the construction of this column was followed. The minicolumn, packed with 400 μ l of Sephacryl S-500, was washed four times with 150 μ l of TE and dried by centrifugation at 600 x g for 2 min, 800 x g for 3 min. The DNA was eluted by centrifugation at 600 x g for 2 min.

2.4 DNA purification by phenol extraction

After Sephacryl purification, the sample volume was adjusted to 400 μ l with double distilled H₂O. One volume of phenol/ chloroform/ isoamyl alcohol (25/24/1, v/v/v) was added and mixed until an emulsion was formed. The mixture was centrifuged at 15000 x g for 3 min and the upper phase was recovered and treated in the same way with one volume of chloroform (Sambrook *et al.*, 2001).

2.5 Ethanol precipitation

Immediately after the phenol extraction, 3 M sodium acetate, pH 5.2 was added to achieve a final concentration of 0.3 M. Exactly 2 volumes of ice-cold absolute ethanol was added and the mixture was incubated at -20°C for 30 min and centrifuged at $15000 \times g$ at 4°C for 10 min. The supernatant was discarded and the pellet washed with 0.5 ml of 70% ice- cold ethanol. The pellet was air dried and dissolved in 100 μl of TE (Sambrook *et al.*, 2001).

2.6 Quantification of DNA

The DNA extracted using the Miller method was quantified using a ND-1000 Nanodrop spectrophotometer (Nanodrop Technologies). An estimated value of the purity of the DNA was determined using the ratio between the readings at 260 nm and 280 nm ($\text{OD}_{260}/\text{OD}_{280}$). The reading at 260 nm was used to calculate the concentration of the DNA in the sample (Sambrook *et al.*, 2001). The purified DNA was diluted with sterile water to 20, 5 and 1 ng/ μl .

2.7 Agarose gel electrophoresis

Agarose gels of 0.8 to 2.5 % (w/v) (Whitehead Scientific) containing 500 $\mu\text{g}/\text{ml}$ ethidium bromide were prepared using standard protocols. Prior to electrophoresis, samples were mixed with 6 X DNA loading dye (Tris-HCl, pH 7.6, 0.03 % bromophenol blue, 60 % glycerol and 0.5 M EDTA, pH 8). *Pst*I-restricted λ -DNA or DNA ladders (Fermentas SM 1108 or 1128) were used as molecular weight marker. Gels were electrophoresed in 0.5 X TBE buffer (45 mM Tris-Cl, 1 mM boric acid,

1mM EDTA, pH 8.0) at a voltage range of 85-100 V for an hour using a GNA 100 electrophoresis apparatus, from Amersham Bioscience (Uppsala Sweden). Ethidium bromide stained agarose gels were visualised under ultraviolet light (300 nm), and photographed with a digital imaging system (AlphaImager 2000, Alpha Innotech, San Leandro, CA).

2.8 Identification of δ -Proteobacteria

2.8.1 Design of oligonucleotide primers

A group-specific oligonucleotide was designed based on the determination of the most conserved regions in the 16S rRNA genes of members of the *Syntrophobacterales*. The G+C content, melting temperature, and length of the primers were chosen to ensure that they met the essential criteria for optimal PCR primers. The G+C content of the primers was 45 %. Aligned sequences were retrieved from Ribosomal Database Project-II (RDP-II) (Cole *et al.*, 2003) databases (GenBank/EMBL/) using the *Hierarchy Browser* sequence search tool. The alignments of sequences were performed using the multiple alignment program CLUSTAL W (Thompson *et al.*, 1994).

2.9 Polymerase Chain Reaction (PCR)

2.9.1 PCR optimization

PCR reactions using primers specific for *Syntrophobacterales* were performed in a final volume of 50 μ l containing 25 ng of metagenomic DNA, primer Syn682F in combination with primer Syn1196R (Table 2.1). Other reaction conditions were as

described in Section 2.9.2. Melting temperatures (T_m) were calculated using the formula: $T_m = 4(G+C) + 2(A+T) ^\circ C$. In order to find the correct annealing temperature for this pair of primers, gradient PCRs were carried out in an Eppendorf Mastercycler® and Mastercycler® gradient (Eppendorf) at a gradient of temperatures (55 to 72°C). Subsequently, amplifications were performed in an automated thermal cycler (Thermo Hybaid system) according to the conditions shown in Table 2.2.

2.9.2 PCR amplification

Table 2.1. Primers used in PCR experiments of this study

Primer	Position	Sequence 5' → 3'	Specificity	Reference
E9F	9-28 ^a	GAGTTTGATCCTGGCTCAG	universal bacteria	Hansen <i>et al.</i> , 1998
U1510R	1510-1492 ^a	GGTTACCTTGTTGTTACACTT	Prokaryotes	Baker <i>et al.</i> , 2003
M340F	340-357 ^a	CCCTACGGGGCGCAGCAG	methanogenic <i>archaea</i>	Watanabe <i>et al.</i> , 2004
M340F-GC ^b	340-357 ^a	CCCTACGGGGCGCAGCAG	methanogenic <i>archaea</i>	Watanabe <i>et al.</i> , 2004
M707R	707-691 ^a	GGATTACAR ^c GATTCAC	methanogenic <i>archaea</i>	Watanabe <i>et al.</i> , 2004
pUCM13F	598-615 ^d	GTTTTCCAGTCACGAC		Farely <i>et al.</i> , 1995
pUCM13R	734-751 ^d	CAGGAAACAGCTATGAC		Reysenbach <i>et al.</i> , 1995
Syn682F	682-701 ^a	GGTGTAGAGGTGAAATTCGT	<i>Syntrophobacterales</i> if used with reverse	This study
Syn1196R	1215-1196 ^a	CATAAAGGCCATGAGGACTT	<i>Syntrophobacterales</i> if used with forward	This study

^a According to *E. coli* 16S rRNA

^b GC clamp CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGG added to 5' end of primer M340F for DGGE analysis

^c R, G or A

^d Position in relation to pTZ57R

Table 2.2. PCR conditions used in this study

Primer Set	Annealing temperature ^e	Extension time (s)
E9F and U1510R	52	120
M340F and M707R	53	60
M340F-GC ^b and M707R	53	60
pUCM13F and pUCM13R	50	60
Syn682F and Syn1196R	65	60

^e All PCRs had an initial denaturation time of 3 min at 94°C, followed by 30 cycles except for the primer set (M340F-GC^b and M707R) which had 35 cycles of 30s at 94°C, 30 s at a specified annealing temperature (in °C), and 72°C at the specified extension time, with final 10 min. For primer set E9F and U1510R, the extension was at 72°C.

PCRs were performed using primers E9F and U1510R, M340F and M707R, and Syn682F and Syn1196R (Table 2.1) with an automated thermal cycler (Thermo Hybaid system) or thermal cycler (Applied Biosystems). For routine PCR assays, unless otherwise specified, PCR reactions (50 µl) contained: metagenomic DNA (20 ng, 5 ng and 1 ng) of different concentrations (20 ng/µl, 5 ng/µl and 1 ng/µl), deoxynucleoside triphosphates (200 µM), 1 X buffer (200 mM Tris-HCl, 100 mM KCl, 100 mM (NH₄)₂SO₄, 200 mM MgSO₄, 1 % (w/v) Triton X-100, pH 8.8), 0.5 µM of each primer (Table 2.2) and 1.25 U *Taq* DNA polymerase (1.5 µl). Conditions used in this study are shown in Table 2.2. Negative controls with no DNA template were included in all PCR experiments. PCR products were separated by agarose gel electrophoresis as described in section 2.7.

For colony PCR, a single fresh colony (2-3 mm in diameter) from an LB plate was picked, transferred into a 0.6 ml microcentrifuge tube and suspended in 40 µl of TE. The mixture was heated in a PCR machine at 99°C for 3 min and centrifuged at

maximum speed (15000 x g) for 2 min. The supernatant was transferred to a new microcentrifuge tube. The supernatant (10 µl) was then used as a template for PCR amplification using primers pUCM13F and pUCM13R (Table 2.1). Colony PCR specific for *Syntrophobacteriales* was performed using Syn682F and Syn1196R.

2.10 Cloning of PCR products in *E. coli* and transformation using pTZ57R/T vector

Bands were excised from agarose gels with a clean sharp scalpel and purified using the Cleanmix Kit (Talent # TA050CLN) or EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Inc.) according to the manufacturer's instructions. The purified 16S rDNA PCR products were ligated into the pTZ57R/T vector using T4 DNA ligase of the Ins T/A clone PCR Product Cloning Kit (#K1214, Fermentas) according to the manufacturer's instructions. Ligations were subsequently transformed into chemically competent *E. coli* strain DH5 α and plated on LB agar plates supplemented with 100 µg/ml ampicillin, 100 µl of 100 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and 20 µl of 50 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) for blue/white screening of recombinants. A tube containing 0.5 µl uncut plasmid was added in order to determine the transformation efficiency of the competent cells. Competent cells from -80°C storage, were placed on ice until thawed and mixed gently by flicking the tube. 50 µl of competent cells was transferred into each tube to the tube containing the uncut plasmid, 100 µl of cells was added (Sambrook *et al.*, 2001).

The contents of the tubes were mixed and placed on ice for 20 min, then heat-shocked for 50 s in a 42°C water bath. The tubes were returned to ice immediately for 2 min. Approximately 950 µl SOC medium (2.0 g bacto[®]-tryptone; 0.5 g bacto[®]-yeast extract; 1 ml of 1 M NaCl; 0.25 ml of 1 M KCl, 1 ml of 2M MgCl₂ stock and 1 ml of 1 M glucose) was added to tubes containing cells transformed with ligation reaction and 900 µl to the tube containing cells transformed with uncut plasmid. The plates were incubated for 1.5 h at 37 °C in a shaker at 30 x g. 100 µl of each transformation culture was plated and incubated overnight at 37°C (Gallenkamp). The plating procedure was done in duplicate (Sambrook *et al.*, 2001).

2.11 Enzymes used in the study

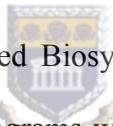
Enzymes were selected according to analysis using WatCut, an on-line tool for restriction analysis, silent mutation scanning, and SNP-RFLP analysis (<http://watcut.uwaterloo.ca/>).

2.12 Amplified rDNA Restriction Analysis (ARDRA)

Amplified rDNA restriction analysis (ARDRA) (Martin-Laurent *et al.*, 2001; Sambrook *et al.*, 2001) of PCR amplicons containing the expected insert size (367 bp) involved *AluI* (4 bp cutter with recognition site 5' AG↓CT 3'), *HaeIII* (4 bp cutter with recognition site 5' GG↓CC 3'), and *RsaI* (4 bp cutter with recognition site 5' GT↓AC 3'), restriction endonuclease digestion (Fermentas). PCR amplicons containing the expected insert size (514 bp) involved *MvaI* (5 bp cutter with recognition site 5' CC↓AGG 3'), *HpyF10VI* (with recognition site 5' GC (N5)

↓NNGC 3') and *SduI*, (with recognition site 5' GDGCH↓C 3') restriction endonuclease digestion (Fermentas). The mixture (20 µl) contained 100 ng DNA, 1 U of restriction endonuclease with its appropriate 10 X Tango buffer (Fermentas) and sterile water. The mixtures were incubated overnight at 37°C (Gallenkamp) and the reaction was terminated by adding 5 µl of 6 X DNA loading dye and separation of products were visualised on a 2.5 % (w/v) agarose gel.

2.13 DNA sequence and analysis

Cloned products were sent to Inqaba Biotech (Pretoria, South Africa) for sequencing. Alternatively, the nucleotide sequences were determined with an automated DNA sequencer model 373A, and a dideoxy chain termination procedure with fluorescein-labeled primers (Perkin Elmer Applied Biosystems) available at the University of Cape Town (South Africa).  Chromatograms were analysed, and the sequences were edited using the BioEdit software (version 7.0.4.1, freeware, Hall, 1999).

Edited sequences were screened against the Genbank database using BLAST (Altschul *et al.*, 1990). The sequences from the GenBank database were aligned using ClustalX version 1.8 (Thompson *et al.*, 1997; Hall, 2001). A neighbour joining phylogenetic tree was constructed from Juke Cantor distances and viewed using Tree View (Page, 1996) and Phylo_win 2.0 (Galtier *et al.*, 1996). Confidence was established by bootstrap analysis with 1000 repetitions (Hall, 2001).

2.14 Storage of clones in liquid media

Approximately 0.5 ml of 50 % sterile glycerol (sterilised by autoclaving for 20 minutes) was added to 1.5 ml of bacterial cultures, vortexed and stored at -80°C for future use.

2.15 Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences obtained in this study have been deposited in GenBank. Accession numbers are shown in Section 3.

2.16 Denaturing Gradient Gel Electrophoresis (DGGE)

PCR was performed using DGGE primers M340F-GC and M707R (Table 2.2). The PCR amplicons were separated on a 1 mm thick 9% polyacrylamide (37.5:1 (w/v) acrylamide: bisacryl, Fluka) gel with a 10-60% denaturant gradient (Biorad 475 gradient former, 100 % UF= 7 M urea, 40 % (V/V) formamide) and a SciPlas (V20-HCDC) apparatus (Table 2.3). 10% (w/v) ammonium persulphate and TEMED (Tetramethylethylenediamine) were added to catalyse the polymerisation process. Electrophoresis was carried out at 60°C for 16 h at 100 V. The gel was stained in 0.5 µg/ml ethidium bromide in 1X TAE for 10 min, destained in 1X TAE (40 mM Tris, 40 mM acetic acid, 1 mM EDTA; pH 7.4) for 15 min, immediately viewed under ultraviolet light (300 nm) and photographed with a digital imaging system (AlphaImager 2000, Alpha Innotech, San Leandro, CA).

Table 2.3. Constituents of 9% acrylamide gels used for DGGE

Constituent	Low denaturant (10%)	High denaturant (60%)
40% Acrylamide	4.5 ml	4.5 ml
50 X TAE	0.4 ml	0.4 ml
Formamide	0.8 ml	4.8 ml
10% ammonium persulphate	0.16	0.16
TEMED	0.016 ml	0.016
Urea	0.84g	5.04 g
Total	20 ml	20



3. Results

3.1. DNA isolation from winery wetland soil

3.1.1. Comparison of metagenomic DNA extraction procedures

Three DNA extraction methods (according to Zhou (1996); Miller (1999) and the FastDNA Spin Kit (Bio101 unit) were used in this study to determine the most efficient method for wetland soil samples. The DNA recovered using the Zhou method had a low molecular mass which could have resulted from shearing, and the FastDNA Spin Kit produced DNA of poor quality that was sheared extensively (data not shown). The Miller method was found to be appropriate for these samples, giving DNA of high molecular mass with little evidence of shearing (Fig. 3.1); this is critical as the use of sheared DNA increases the occurrence of chimeric 16S rDNA amplicons during PCR (Stach *et al.*, 2001). The sodium dodecyl sulfate (SDS) included in the Miller protocol limits the extraction of humic substances and shearing of DNA (Roose-Amsaleg *et al.*, 2001).

DNA extracted from the lower core and upper core samples was of high molecular weight but high levels of RNA were co-extracted from upper core samples, necessitating an RNase treatment. The samples collected also contained fibrous plant material, especially the upper core samples. Sample pre-treatment with the aid of a 2 mm diameter sieve was therefore introduced in order to exclude all plant material from the samples. The sieving and centrifugation did not improve the quality of the DNA, but improved the yield of DNA when compared to the unprocessed sample, since a considerable amount of water was removed with the centrifugation step.

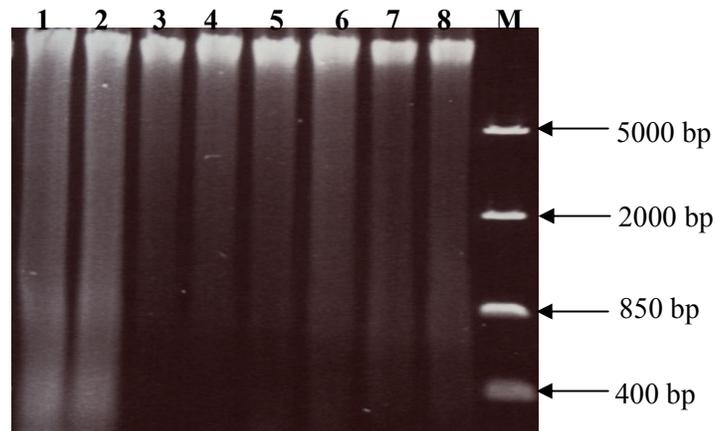


Fig. 3.1. Agarose gel (0.8%) of total DNA extracted from lower core and upper core soil samples using the modified Miller method. Lanes: M, DNA ladder; 1-2, upper core samples (0-5 cm in depth); 3-8, lower core samples (5-15 cm in depth).

3.1.2. Purifications of DNA

Purification of crude DNA extracted from soil is necessary because it contains humic and fulvic acids that are inhibitors of the enzyme *Taq* DNA polymerase (Kuske *et al.*, 1998; Roose *et al.*, 2001). The humic acids and phenolic compounds in sediment are difficult to remove, making the DNA purification step very crucial (Miller *et al.*, 1999). Humic acids have physicochemical properties similar to those of nucleic acids, so they are easily co-extracted with DNA. The presence of the humic acids (brownish colour) was noted in the all crude DNA samples, especially from lower core samples. The level of contaminants decreased considerably after the PVPP purification step. With the exception of one sample (lanes 1, 4, and 7), the PVPP and Sephacryl minicolumn purification methods caused a minor loss of DNA, as represented in Fig. 3.2.

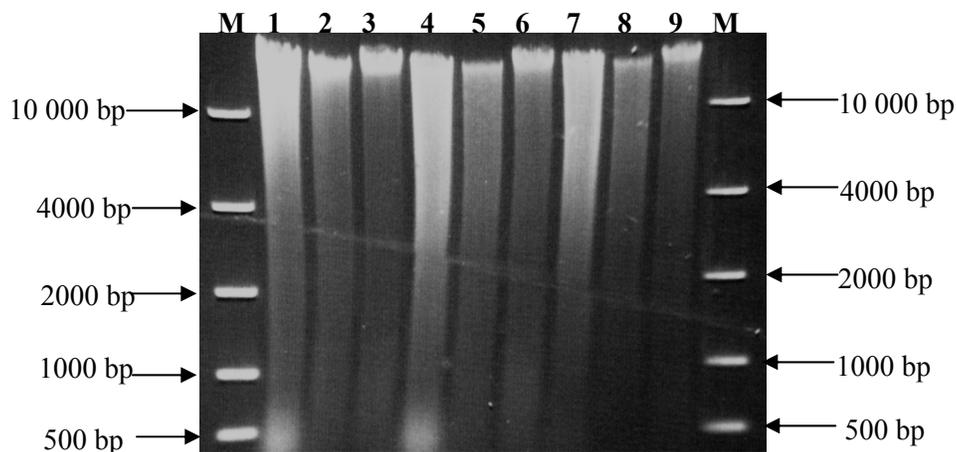


Fig. 3.2. Agarose gel (0.6%) of total DNA extracted from soil samples using the modified Miller method. Lanes: M, DNA ladder in base pairs; 1, 2 and 3; crude DNA from upper core samples (0-5 cm in depth) and lower core samples (5-15 cm in depth), respectively; 4, 5 and 6, DNA from upper core and lower core samples after PVPP minicolumns purification; 7, 8 and 9, DNA from upper core and lower core samples after Sephacryl minicolumns purification.



3.1.3. Quantification of DNA and purity analysis

The DNA was quantified using a spectrophotometer and its purity was determined using A_{260}/A_{280} absorbance ratios. Low A_{260}/A_{280} ratios (<1.7) indicate protein contamination, whereas low A_{260}/A_{230} ratios (<2.0) indicate humic acid contamination (Stach *et al.*, 2001). For some unknown reason, the DNA yield in the upper core sample increased after the RNase treatment. Results represented in Table 3.1 indicate that none of the purification methods removed all the contamination since the A_{260}/A_{230} ratios were <2.0 . For optimum purity, an additional step involving hexadecyltrimethylammonium bromide (CTAB) combined with Sephadex G200 column purification could be applied (Miller *et al.*, 1999; Roose-Amsaleg *et al.*, 2001). Concentration measurements of crude and partially purified DNA were

probably inaccurate because humic substances absorb at 260 nm. The DNA purity for most samples improved after PVPP and Sephacryl purification.

Table 3.1. Quantification of nucleic acids

Sample ID	A260/280	A260/230
Upper core sample C ^a	1.7	2
Upper core sample R ^b	1.45	1.69
Upper core sample P ^c	1.71	2.08
Upper core sample S ^d	1.71	2.27
Lower core sample C ^a	1.68	1.84
Lower core sample R ^b	1.66	1.72
Lower core sample P ^c	1.69	1.97
Lower core sample S ^d	1.72	2.79
Lower core sample C ^a	1.36	0.81
Lower core sample R ^b	1.34	0.71
Lower core sample P ^c	1.44	1.17
Lower core sample S ^d	1.64	1.8

^a Crude DNA samples

^b DNA after RNase treatment

^c DNA after PVPP minicolumn purification

^d DNA after Sephacryl minicolumn purification, phenol extraction and ethanol precipitation

3.1.4. PCR amplification

Amplification of 16S rDNA was successfully performed using universal bacterial primers, E9F and U1510R, confirming the presence of high purity DNA. The DNA extracted and purified from the wetland soil was tested as template in PCR amplifications at different concentrations (1 to 20 ng per reaction) to determine the effects of different template concentrations on the amount of PCR product obtained. Most samples gave positive results for all concentrations (Fig. 3.3). Failure to amplify

could indicate that the lower core sample was not sufficiently purified and contained traces of humic acids that are inhibitors of the *Taq* DNA polymerase. Fig. 3.3 shows that the PCR products obtained had the expected size of 1.5 kb and produced clear distinctive bands. Based on these results, all further PCR reactions were performed with 1-5 ng wetland soil DNA.

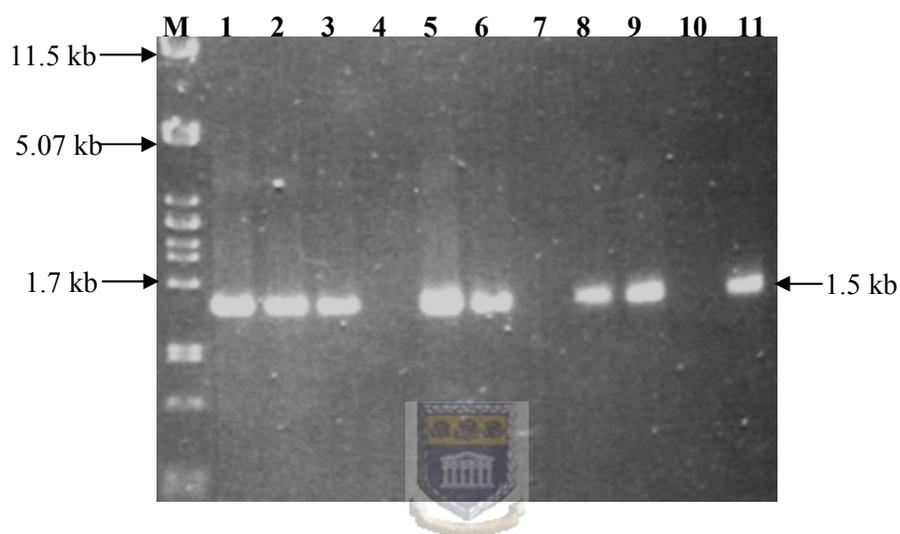


Fig. 3.3. PCR products generated from purified DNA using different amounts of template with E9F and U1510R primers. Lanes: M, *Pst*I-restricted λ -DNA; 1, 2 and 3, upper core sample, 20 ng, 5 ng and 1 ng respectively; 4, 5 and 6, lower core sample, 20 ng, 5 ng and 1 ng respectively; 7, 8 and 9, lower core sample, 20 ng, 5 ng and 1 ng respectively; 10, negative control; 11, positive control, *Vibrio cholerae* DNA.

3.2. Methanogenic *Archaea* in winery wetland soil

Molecular approaches were chosen for identifying methanogens because (a) these methods provide a rapid and efficient means of identifying multiple genospecies, and (b) growing methanogens is difficult.

3.2.1. PCR amplification of 16S rDNA

The PCR specific for methanogens using primers M340F and M707R was successful. Fig. 3.4 shows that the PCR products obtained from methanogenic primers had the expected size of 367 bp and produced clear distinctive bands.

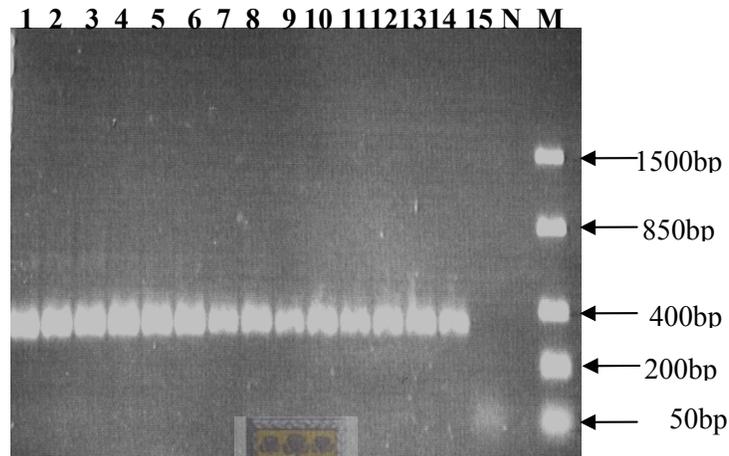


Fig 3.4. PCR products generated from purified DNA from upper core and lower core samples. PCR products were electrophoresed on a 1.25% agarose gel. Lanes: 1-7, lower core and upper core sample, 20 ng; 8-14, upper core and lower core sample, 5 ng; M, DNA ladder; 15, *Escherichia coli*; N, negative control without DNA.

3.2.2. 16S rDNA clone library and Amplified rDNA Restriction Analysis

(ARDRA)

PCR products were ligated into pTZ57R/T vector, and transferred into *E. coli* strain DH5 α . After transformation, blue-white screening allowed 60 white colonies to be randomly selected for determining the length of the insert by colony PCR. Results from colony PCR showed that 45 of the white colonies (75%) contained a 413 bp insert (Fig. 3.5). The remaining 15 white colonies (25%) contained recombinant plasmids with small inserts or religated vectors (Table 3.2).

Table 3.2. Colony counts after transformation using pUCM13F and pUCM13R primers

Transformation Plates	No. of white colonies	No. of blue colonies
Lower core sample	85	20
Upper core sample	70	25
Positive control (pUC 18)	3	TNTC
Control	65	30
Background control	7	83

TNTC. Too Numerous To Count.

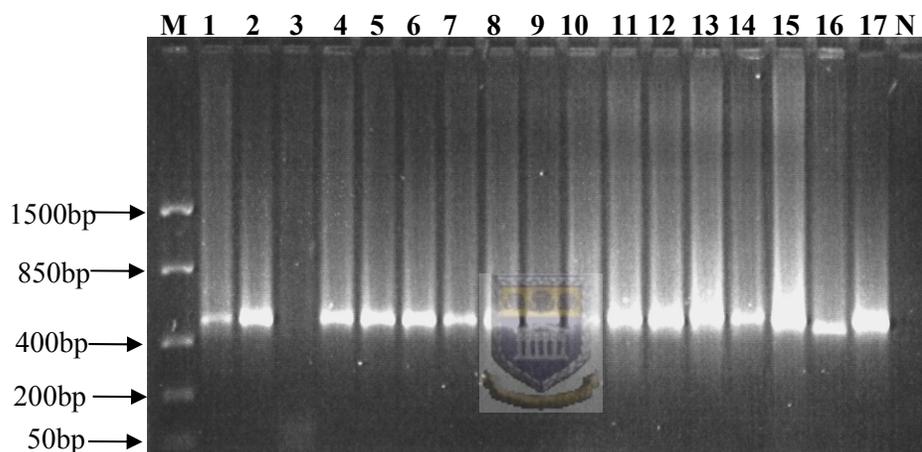


Fig. 3.5. Colony PCR using pUCM13F and pUCM13R primers. Representatives of PCR products obtained from colonies randomly chosen from the methanogenic 16S rDNA clone library of the upper core and lower core samples. Lanes: M, DNA ladder; 1-17, white colonies from the lower core and upper core sample; N, negative control without DNA.

The 45 colony PCR amplicons were subjected to ARDRA in order to assess the variety of DNA sequences in the library. Resolution of ARDRA patterns requires two or more restriction enzymes (Dunbar *et al.*, 1999; Myer *et al.*, 1996). The three tetranucleotide specific restriction enzymes, *AluI*, *HaeIII* and *RsaI*, used in this study provided detailed banding patterns on digestion of the inserts. *FokI* (a 6 bp cleavage enzyme with recognition site 5'GGATG (N₉) ↓ 3') was also used in this study but

showed no restriction of the DNA. Fig. 3.6a and Table 3.3a show that the *AluI* restriction endonuclease digestion gave the best resolution, showing 7 distinct cleavage patterns. Digestion pattern A was dominant in both samples (67%), while pattern B only occurred in the lower core sample and patterns E, F, and G occurred only in upper core sample (Table 3.3a). The *AluI* digestion of 413 bp PCR fragment produced 213 bp, and 199 bp fragments, indicating a single cleavage site. The patterns in lane 3 and 4 contain a 200 bp fragment only, suggesting that the other fragments were too small to be viewed on the agarose gel.

The patterns from *HaeIII* and *RsaI* restriction endonuclease digestion gave 6 distinct and 4 cleavage patterns, respectively. Digestion pattern A was dominant in both samples for *HaeIII* and *RsaI* (69% and 82%, respectively), while digestion pattern C only occurred in the lower core sample. Pattern D only occurred in the upper core sample. For *RsaI*, patterns, E, and F occurred in the upper core sample. The PCR fragment (413 bp) from *HaeIII* digestion produced 190, 90, 80 and 50 bp fragments, (lanes 1, 2, 5-13). The digestion pattern shown in lanes 3 and 4 (Fig. 3.6b) produced 220 and 190 bp fragments. In some instances DNA was not digested, presumably because there was no *RsaI* site in the DNA amplicons (lanes 3, 4 and 8) (Fig. 3.6c).

In total, 60 clones, 30 from the lower core sample and 30 from the upper core sample, were analysed. Of these, twenty-two showed unique restriction patterns. More unique patterns were observed within the lower core sample (59%) than within the upper core sample (41%).

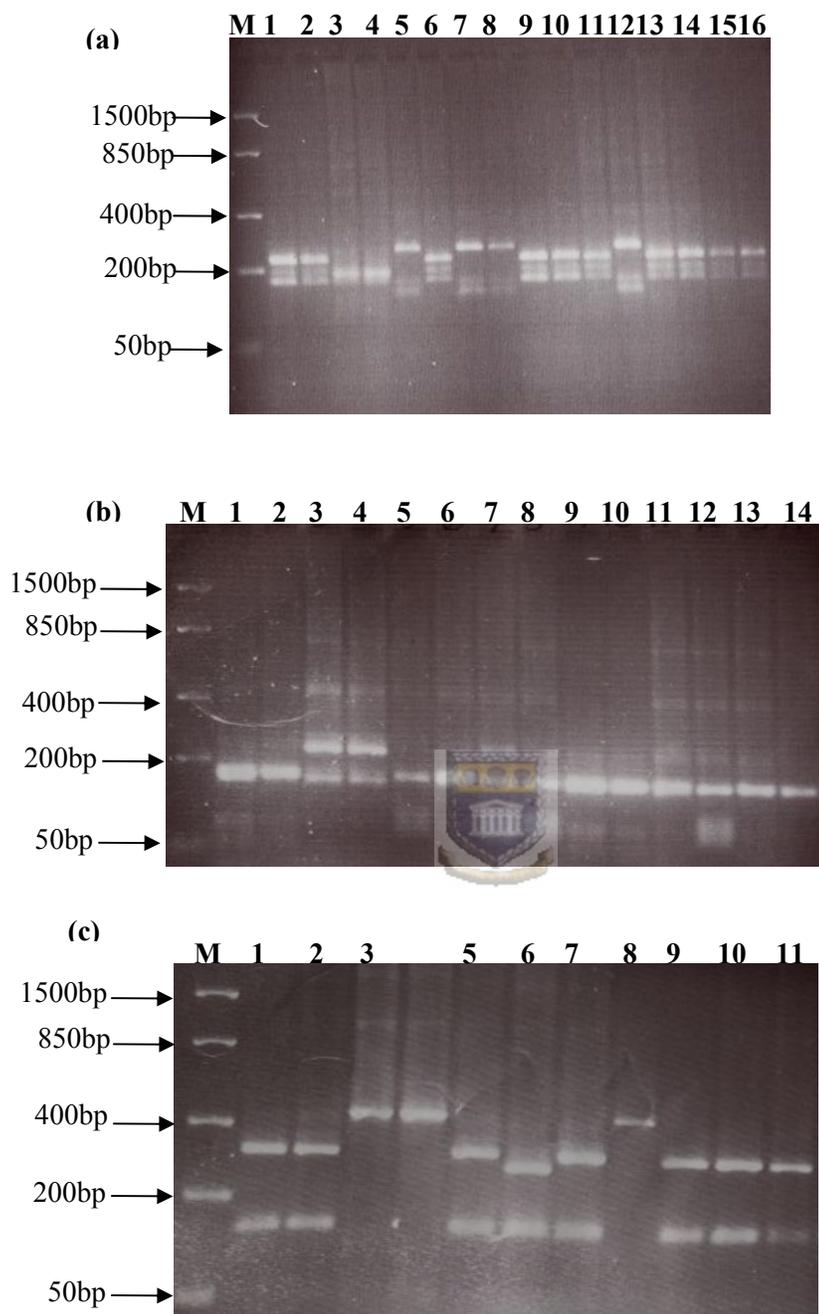


Fig. 3.6. ARDRA patterns of 16S rDNA PCR products of methanogenic archaea using pUCM13F and pUCM13R primers. **a-c:** 16S rDNA amplicons of colonies from lower core and upper core samples digested by *AluI*, *HaeIII* and *RsaI*, respectively, separated on a 2.5% agarose gel. Lanes: M, DNA ladder; 1-16, PCR products obtained from white colonies chosen randomly from lower core and upper core samples.

From each unique restriction pattern, a representative clone was sequenced (Table 3.3).

Table 3.3 a. Summary of ARDRA patterns from 16S rRNA genes of methanogenic archaea using *AluI* restriction endonuclease digestion

Pattern	Clones from Lower core sample	Clones from Upper core sample
A	B1, B3, B7, B11, B12, B13, B15, B16, B17, B18, B19, B20, B21, B22, B24, B25, B27, B28	T1, T14, T15, T18, T19, T20, T21, T23, T25, T27, T29, T30
B	B4, B5, B14, B23, B30	N/A
C	B6, B9, B10	T2
D	B26	T28
E	N/A	T17
F	N/A	T22
G	N/A	T24, T26

N/A. not applicable

B clones that were selected from lower core sample

T clones that were selected from upper core sample



Table 3.3 b. Summary of ARDRA patterns from 16S rRNA genes of methanogenic archaea using *HaeIII* restriction endonuclease digestion

Pattern	Clones from Lower core sample	Clones from Upper core sample
A	B1, B3, B7, B11, B12, B13, B15, B16, B17, B18, B19, B20, B21, B22, B23, B24, B25, B27, B28, B30	T1, T14, T18, T20, T21, T22, T23, T24, T25, T27, T30
B	B4, B5	T17, T19, T28
C	B6, B9, B10, B14	N/A
D	B26	T2, T29
E	N/A	T15
F	N/A	T26

N/A. not applicable

B clones that were selected from lower core sample

T clones that were selected from upper core sample

Table 3.3 c. Analysis of ARDRA patterns from 16S rRNA gene of methanogenic archaea using *RsaI* restriction endonuclease digestion

Pattern	Clones from Lower core sample	Clones from Upper core sample
A	B1, B3, B6, B9, B11, B12, B13, B14, B15, B16, B17, B18, B19, B20, B21, B22, B23, B25, B27, B28, B29, B30	T1, T14, T15, T17, T18, T19, T20, T21, T22, T23, T24, T25, T27, T28, T30
B	B4, B5, B10	T2, T29
C	B7, B24	N/A
D	N/A	T26

N/A not applicable

B clones that were selected from lower core sample

T clones that were selected from upper core sample

3.2.3. Methanogenic 16S rDNA sequence analysis

The vector and primer regions of selected amplicons DNA sequences were excluded prior to BLAST analysis. Sequences length ranged from 254 to 310 nucleotides. All the sequences retrieved fell within the *Euryarchaeota* subdomain (Table 3.4) except for the sequence of clone T26 which was identified as a member of the domain *Bacteria*. Most of the sequences retrieved (82%) were relatively close to sequences of uncultured clones from environmental samples and relatively few (18%) showed high homology to known methanogenic species. Only the sequence of clone T1 exhibited more than a 5% divergence from sequences in the GenBank database, suggesting that this clone belonged to a different and possibly novel genus (<95% identity to those in the NCBI database).

Several sequences were nearly identical to an uncultured *Methanomicrobiaceae* clone (36%), an uncultured *Methanosaetaceae* clone (23%) and an uncultured *Methanosaeta* clone (14%). The divergence of the clones from these three sequences

did not exceed 2% with the exception of clones B24, B4, and T1, suggesting that they belonged to the same species (97 or 97.5% identity to those in the NCBI database). The diversity of methanogenic *Archaea* in this wetland was confirmed by the results represented in Table 3.4 using BLAST analysis and sequencing of 16S rDNA.

Table 3.4. Blast analysis of 16S unique rDNA insert sequences

Clone	% Identity	Nearest Organism match	Accession Number
B1	99	Uncultured <i>Methanomicrobiaceaea</i> archaeon	AY133896
B4	97	Uncultured <i>Methanomicrobiaceaea</i> archaeon	AY133896
B6	98	Uncultured <i>Methanosaeta</i> sp.	AY780569
B7	99	Uncultured <i>Methanomicrobiaceaea</i> archaeon	AY125717
B9	98	Uncultured <i>Methanosaetaceae</i> archaeon	AY125710
B11	98	methanogenic endosymbiont of <i>Caenomorpha</i> sp. 2	AJ132652
B14	100	Uncultured <i>Methanosaetaceae</i> archaeon	AY133932
B17	97	Uncultured <i>methanogenium</i> sp.	AY177809
B23	99	Uncultured <i>Methanosaeta</i> sp.	AY780569
B24	96	Uncultured <i>Methanomicrobiaceaea</i> archaeon	AY133924
B26	99	Uncultured <i>Methanomicrobiaceaea</i> archaeon	AY133896
B27	98	Uncultured <i>Methanomicrobiaceaea</i> archaeon	AY133896
B30	99	Uncultured <i>Methanosaetaceae</i> archaeon	AY133932
T1	93	Uncultured <i>Methanomicrobiaceaea</i> archaeon	AY133922
T2	98	methanogenic endosymbiont of <i>Caenomorpha</i> sp. 2	AJ132652
T14	98	Uncultured <i>Methanosaeta</i> sp.	AY780569
T15	98	Uncultured <i>Methanomicrobiaceaea</i> archaeon	AY133901
T17	99	<i>Methanobacterium</i> sp. IM1	AY274451
T22	97	Uncultured <i>Methanosaetaceae</i> archaeon	AY125710
T24	98	Uncultured <i>Methanosaetaceae</i> archaeon	AY133916
T26	97	Uncultured bacterium	AY134895
T28	100	<i>Methanobacterium</i> sp. IM1	AY274451

Figure 3.7 shows the phylogenetic tree of the methanogenic 16S rDNA sequences from wetland soil of the Middelvlei winery. Sequences of known methanogen species retrieved from the Genbank database were added to the tree in order to allocate the experimental clusters to the specific taxonomic groups. The neighbour-joining phylogenetic tree (Fig. 3.7) include the four orders of the *Euryarchaeota*, namely *Methanomicrobiales*, *Methanosarcinales*, *Methanobacteriales*, and *Methanococcales*. Clone T26 belongs to the Domain *Bacteria*, and was used as an outgroup. The majority of sequences belonged to one of two clusters. Cluster *Methanomicrobiales*, and *Methanosarcinales* compromised 50% and 37% of the total sequences, respectively. The remaining sequences clustered with the *Methanobacteriales* (9%). Most of the *Methanomicrobiales* sequences were closely related to sequences from cultured members of the *Methanomicrobiaceae*, cluster *Methanosarcinales* sequences were mostly related to sequences from *Methanosaetaceae*, while *Methanobacteriales* sequences were mostly related to those from *Methanobacteriaceae*.

The sequences of clones B26, B1, B4, B7, B17, B24, and T1 (cluster *Methanomicrobiales*) were not closely related to any methanogen sequences accessible by BLAST analysis and had a bootstrap support of 94%. The branch of these clones deeply branched in the tree and could not be affiliated to any known species. It can therefore be concluded that these clones could be related to methanogenic endosymbiont of *Caenomorpha* sp. 2 or a new genus that has not been cultured. Clones, B6; B23; B30; T14; and T24 were grouped with *Methanosaetaceae* (*Methanosarcinales*) and showed close relationship with *Methanosaeta concilii* from

the database. Clones T22 and B9 belong to the *Methanosarcinales* with a 63% bootstrap support. These two clones were closely related to *Methanosaeta concilii*. Clone B14 affiliated with *Methanosaeta* sp. (order *Methanosarcinales*) with a bootstrap of 90%. Clones T17 and T28 (order *Methanobacteriales*) were monophyletic with 100% bootstrap values, and this monophyletic branch clustered with the genus *Methanobacterium* with a 66% bootstrap value.



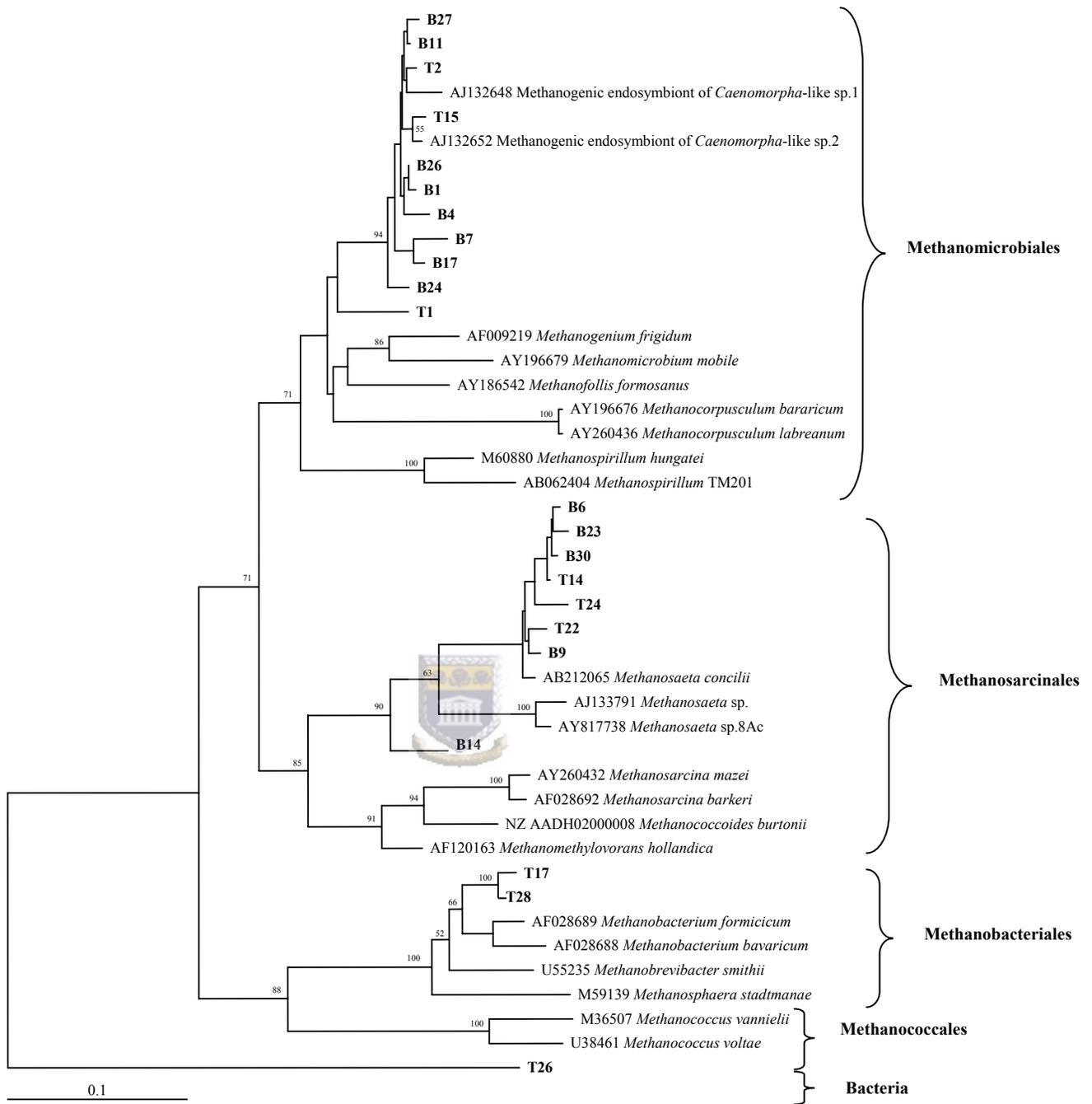


Fig. 3.7. Phylogenetic tree of partial 16S rRNA gene sequences of methanogenic archaea from wetland soil. The scale bar of the tree represents a 10% difference in nucleotide sequences. The sequences obtained in the present study are shown in bold letters. The tree was constructed with Phylo_win 2.0 using the neighbour-joining algorithm. Numbers at nodes represent the percentages of bootstrap resamplings based on 1000 replicates; only values above 50 percent are presented. T26 was used as an outgroup. Accession numbers of sequences are indicated.

3.2.4. Diversity of methanogenic archaea using DGGE analysis

Partial 16S rDNA of methanogenic microorganisms was amplified from wetland soil using primer pair M340F-GC and M707R, and the products separated by DGGE. The resulting bands were compared with those obtained from members of the clone library. The environmental samples from the upper core of the wetland soil, gave distinct 16S rDNA band patterns on DGGE gels and showed good resolution and separation (Fig. 3.8A). The DGGE profiles revealed the presence of eight to eleven well defined bands for the upper core sample. Most bands from the clones corresponded to bands of the environmental soil sample (Fig 3.8). Clones in lanes 1 and 9 corresponded to strong thick bands in environmental sample (Fig. 3.8A), while the rest of the clones corresponded to the faint bands within the environmental sample. It was not possible to analyse clone in lane 4 because the signal was not clear.



The DGGE profiles revealed the presence of approximately fifteen well defined bands for the lower core sample, and bands from all clones corresponded to bands of the environmental soil sample (Fig. 3.8B). The bands that corresponded to strong bands in the environmental sample suggest that the species are dominant within the sample. The lower core sample had substantially more bands compared to the upper core sample, confirming that the methanogen species diversity in the lower sample was significantly greater than the upper core sample.

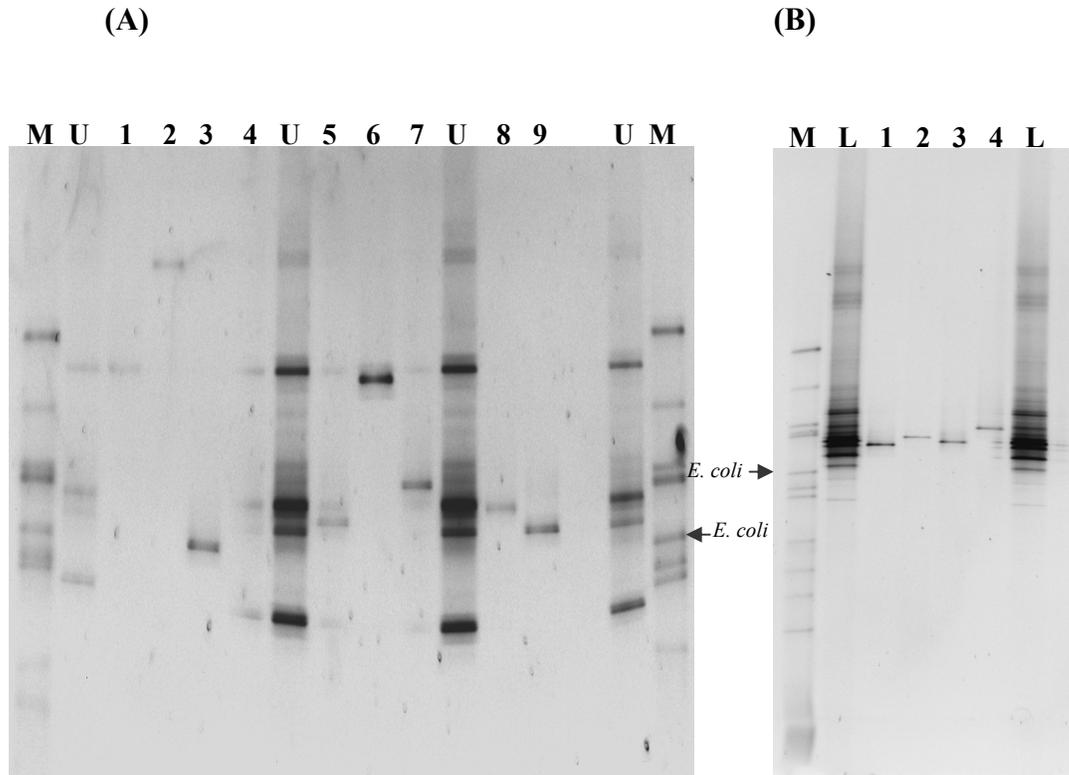


Fig. 3.8. DGGE band patterns of PCR products obtained from wetland soil using M340F-GC and M707R primers (picture negatively converted). **A**, environmental sample and clones from upper core sample. Lanes: M, DGGE marker consisting of products amplified from a mixture of genomic DNAs of 13 clones; 1, clone T1 (mm); 2, clone T2 (mm); 3, clone T14 (ms); 4, clone T15 (mm); U, upper core sample; 5, clone T17 (mb); 6, clone T22 (ms); 7, clone T24 (ms); 8, clone T26 (b), 9, clone T28 (mb). **B**, environmental sample and clones from lower core sample. Lanes: M, DGGE marker consisting of products amplified from a mixture of genomic DNAs of 13 clones; 1, clone B1 (mm); 2, clone B4 (mm); 3, clone B6 (ms), clone B7 (mm). mm=*Methanomicrobiales*; mb=*Methanobacteriales*; ms=*Methanosarcinales*; b=*Bacteria*. The gradient of denaturants was 10-60%.

3.3. Identification of *Syntrophic Bacteria*

3.3.1. Primer design

The primers Syn682F and Syn1196R were designed to be specific for mostly microorganisms belonging to two families of *Syntrophobacteriales*:

Syntrophobacteraceae and *Syntrophaceae*. Syntrophs are secondary fermenters that ferment primary fermentation products, in particular alcohols and long chain fatty acids, to produce H₂, CO₂, and acetate. The H₂ is consumed by methanogens. Syntrophs are taxonomically diverse and are found in many different genera (*Clostridium* and δ -*Proteobacteria*). The focus on these two genera is very important because they provide a 'metabolic' link with coal degradation, since they ferment aromatics as substrates. These primers were found to be well suited for this study because they matched most of targeted groups when analysed using the tools of the Ribosomal Database Project-II (RDP-II). Results from RDP-II show that the forward and reverse primers targeted about 40%, and 36%, respectively, of microorganisms from the class of δ -*Proteobacteria*, largely sequences from species type strains and non-type strains. Other microorganisms that were targeted from the database using both primers were those belonging to class α -*Proteobacteria*, β -*Proteobacteria* and γ -*Proteobacteria*, but all with a <8% value.

The analysis using Syn682F and Syn1196R further indicated the presence of (i) some species of the family *Desulfobacteraceae*, (62% and 33%) for the forward and reverse primer, respectively and *Desulfobulbaceae* (93% and 92%) (ii) bacteria belonging to the family *Syntrophobacteraceae* (53% and 54%), and *Syntrophaceae* (82% and (92%) and (iii) family of *Cystobacteraceae* (24%) and (94%), respectively. Only the forward primer indicated species of family *Polyangiaceae* (94%) and *Haliangiaceae* (100%). The most dominant genera were from the family

“*Syntrophobacteraceae*”, “*Syntrophaceae*”, *Cystobacteraceae*, and among the family of *Desulfobacteraceae* and “*Desulfobulbaceae*” (Table 3.5).

Table 3.5. Analysis showing targeted genera using Syn 682F and Syn 1196 R primers

Order	Family	Genus	Forward Primer %	Reverse Primer %	
<i>Rhodospirillales</i>	<i>Acetobacteraceae</i>	<i>Acidisphaera</i>	4	0	
		unclassified <i>Acetobacteraceae</i>	0	2	
	<i>Rhodospirillaceae</i>	unclassified <i>Rhodospirillales</i>	0	18	
	<i>Rhodobacteraceae</i>	<i>Sulfitobacter</i>	<1	0	
<i>Sphingomonadales</i>	<i>Sphingomonadales</i>	<i>Erythrobacter</i>	0	1	
<i>Rhizobiales</i>	<i>Bradyrhizobiaceae</i>	<i>Bradyrhizobium</i>	<1	0	
<i>Desulfobacterales</i>	<i>Desulfobacteraceae</i>	<i>Desulfobacter</i>	100	0	
		<i>Desulfobacula</i>	84	0	
		<i>Desulfofaba</i>	50	0	
		<i>Desulfofrigus</i>	100	0	
		<i>Desulfomusa</i>	100	0	
		<i>Desulforegula</i>	100	0	
		<i>Desulfosarcina</i>	93	86	
		<i>Desulfospira</i>	100	0	
		<i>Desulfotignum</i>	100	0	
		<i>Desulfococcus</i>	0	80	
		<i>Desulfonema</i>	0	100	
		unclassified <i>Desulfobacteraceae</i>	53	36	
		<i>Desulfobulbaceae</i>	<i>Desulfobulbus</i>	88	87
			<i>Desulfocapsa</i>	92	96
			<i>Desulfofustis</i>	100	67
<i>Desulfohopalus</i>	94		98		
<i>Desulfotalea</i>	100		90		
<i>Nitrospina</i>	0		44		
<i>Desulfuromonales</i>	<i>Desulfuromonaceae</i>	Unclassified <i>Desulfuromonaceae</i>	0	6	
<i>Syntrophobacterales</i>	<i>Syntrophobacteraceae</i>	<i>Syntrophobacter</i>	100	100	
		<i>Desulfacinum</i>		33	
		<i>Desulforhabdus</i>	88	88	
		<i>Desulfovirga</i>	100	67	
		Unclassified <i>Syntrophobacteraceae</i>	35	32	
		<i>Syntrophaceae</i>	<i>Syntrophus</i>	82	91
			Unclassified <i>Syntrophobacterales</i>	80	20
<i>Myxococcales</i>	<i>Cystobacteraceae</i>	<i>Cystobacter</i>	0	95	
		<i>Archangium</i>	100	100	
		<i>Mellittangium</i>	62	100	
		<i>Stigmatella</i>	0	100	
		Unclassified <i>Cystobacteraceae</i>	29	86	
	<i>Myxococcaceae</i>	<i>Myxococcus</i>	0	100	
		<i>Polyngium</i>	100	0	
	<i>Polyangiaceae</i>	<i>Chondromyces</i>	94	0	
	<i>Haliangiaceae</i>	<i>Haliangium</i>	100	0	
		Unclassified <i>Myxococcales</i>	85	5	
		<i>Nannocystaceae</i>	<i>Nannocystis</i>	100	0

3.3.2. PCR amplification of 16S rDNA

Gradient PCR was performed to identify the highest possible annealing temperature. This is important because amplification of non-target 16S rDNA (such as from *E. coli*) had to be prevented. *E. coli* was included as a control to test the specificity of the primer set. The primer combination provided a high yield of specific product (on the basis of gel band intensity) and few of non-specific amplification products over a broad range of annealing temperatures. Non-specific products were not noted in this PCR experiment. Representative results from temperature gradient PCR with temperatures from 55 °C to 72 °C are presented in Fig 3.9 a. These data show clearly that the annealing temperatures between 64.2 °C and 66.5 °C worked well for this PCR reaction, showing no products from the *E. coli* control (lanes 22 to 30).



PCR products were obtained from *E. coli* at annealing temperature lower than 64.2 °C. At temperatures higher than 66.5 °C, PCR was not successful, except for the weak band shown in lane 23 (Fig. 3.9a). A final experiment was performed at 65 °C to confirm that this temperature was optimal for the amplification of this fragment. An excellent amplification was achieved as shown by the intensity of the bands. The PCR products obtained had the expected size of 514 bp (Fig. 3.9b). A cloned 16S rDNA from Middelvlei soil sample, with 91% sequence identity to *Syntrophus gentianae* (plasmid pMid-3h) was included in the experiment as a positive control.

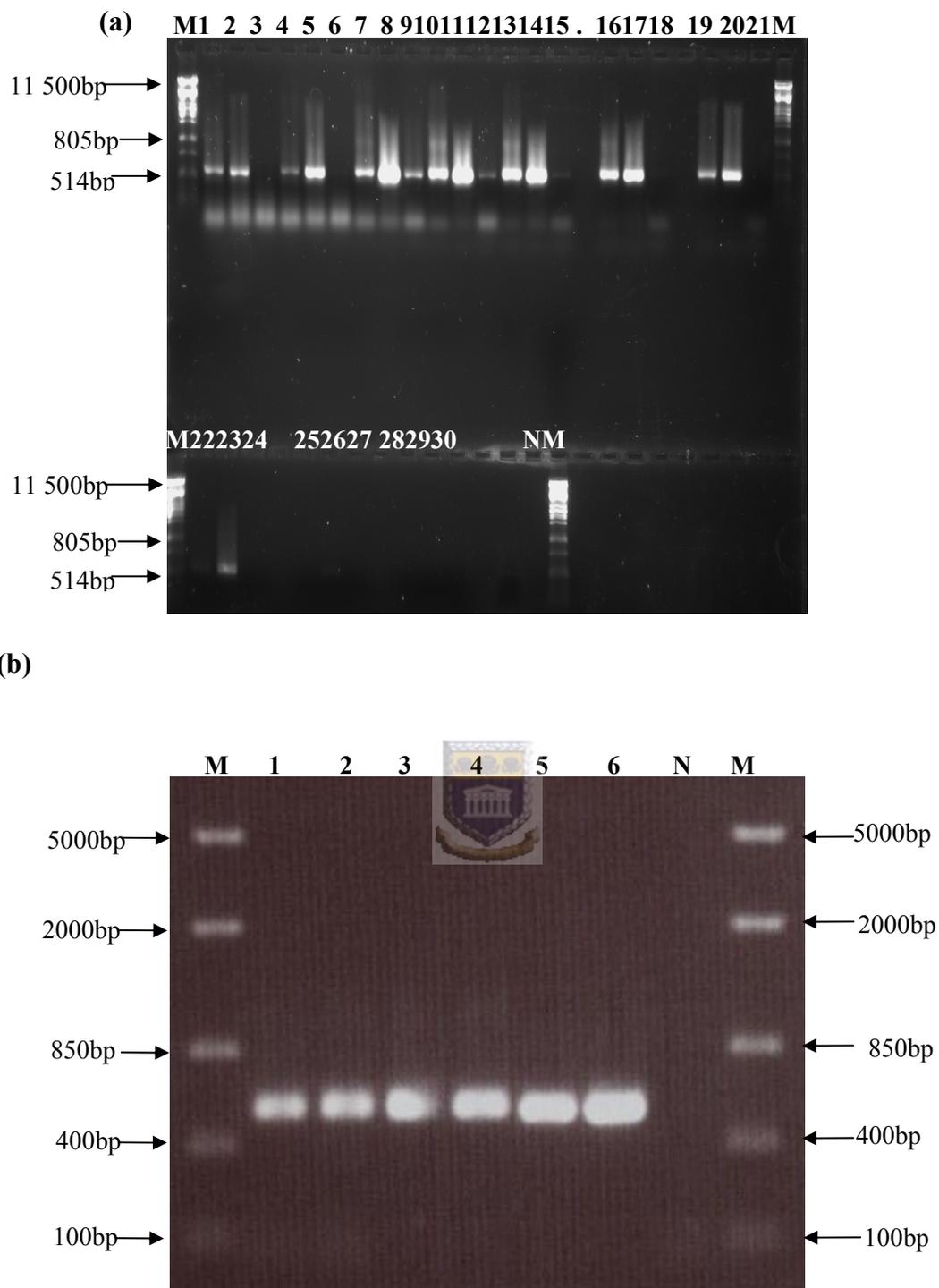


Fig 3.9. Optimisation of PCR for the amplification of syntrophic 16S rDNA using primers Syn682F and Syn1196R. **a**, PCR products were electrophoresed on a 1% agarose gel. Lanes 1-30, gradient PCR, annealing temperatures from 55 °C to 72 °C. Lanes: M, *Pst*I-restricted λ -DNA; 1-3, 25 ng DNA, positive control, *E. coli* at 55 °C respectively; 4-6, 25 ng

metagenomic DNA from wetland, positive control, *E. coli* at 56.5 °C respectively; 7-9, 25 ng metagenomic DNA from wetland, positive control, *E. coli* at 57.8 °C respectively; 10-12, 25 ng metagenomic DNA from wetland, positive control, *E. coli* at 59.7 °C respectively; 13-15, 25 ng DNA metagenomic DNA from wetland, positive control, *E. coli* at 61.9 °C respectively; 16-18, 25 ng DNA, positive control, *E. coli* at 64.2 °C respectively; 19-21, 25 ng metagenomic DNA from wetland, positive control, *E. coli* at 66.5 °C respectively; 22-24, 25 ng metagenomic DNA from wetland, positive control, *E. coli* at 68.7 °C respectively; 25-27, 25 ng DNA, positive control, *E. coli* at 70.5 °C respectively; 28-30, 25 ng metagenomic DNA from wetland, positive control, *E. coli* at 71.8 °C respectively; N, negative control without DNA. **b**: PCR products were at 65 °C and electrophoresed on a 1% agarose gel stained with ethidium bromide. Lanes: M, DNA ladder (Fermentas SM 1108); 1-2, 25 ng of upper core sample; 3-4, 25 ng of lower core sample; 5-6, positive control; 7, *E. coli*.

3.3.3. 16S rDNA clone library and Amplified rDNA Restriction Analysis

(ARDRA)



PCR products were ligated as outlined in section 3.2.2. After transformation, blue-white screening allowed 75 white colonies to be randomly selected for determination of the length of the insert by colony PCR using pUCM13F and pUCM13R primers (Table 3.6). Results of colony PCR showed that 69 white colonies (92%) contained a 700 bp insert (Fig. 3.10). The remaining 6 white colonies (8%) contained recombinant plasmids with small inserts or religated vectors.

Table 3.6. Colony counts after transformation using Syn682F and Syn1196R primers

Transformation Plates	No. of white colonies	No. of blue colonies
Lower core sample	118	28
Upper core sample	130	12
Positive control (pUC 18)	5	TNTC
Control	80	10
Background control	3	95

TNTC. Too Numerous To Count.

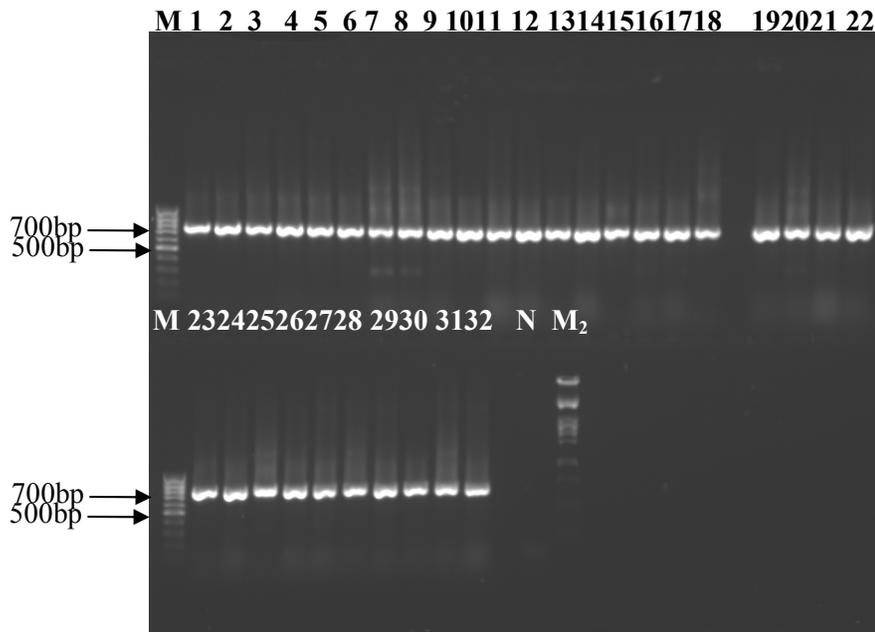


Fig. 3.10. Colony PCR using pUCM13F and pUCM13R primers. PCR products obtained from colonies randomly chosen from the metagenomic 16S rDNA clone library of the upper core and lower core samples. Lanes: M, DNA ladder (Fermentas SM 1108); 1-32, white colonies from the lower core and upper core sample; N, negative control without DNA; M₂, *Pst*I-restricted λ -DNA.

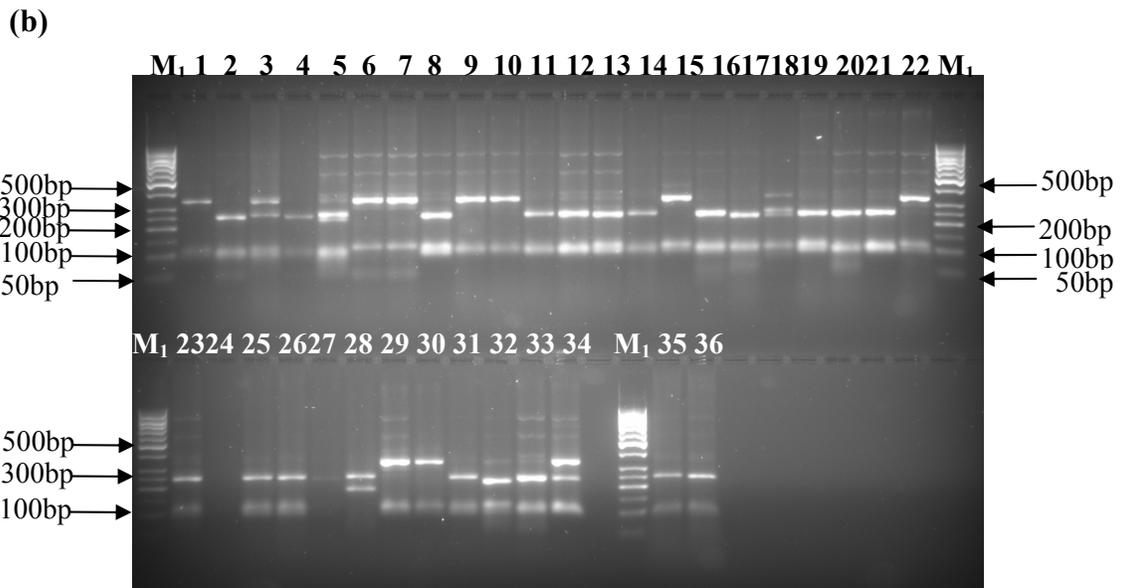
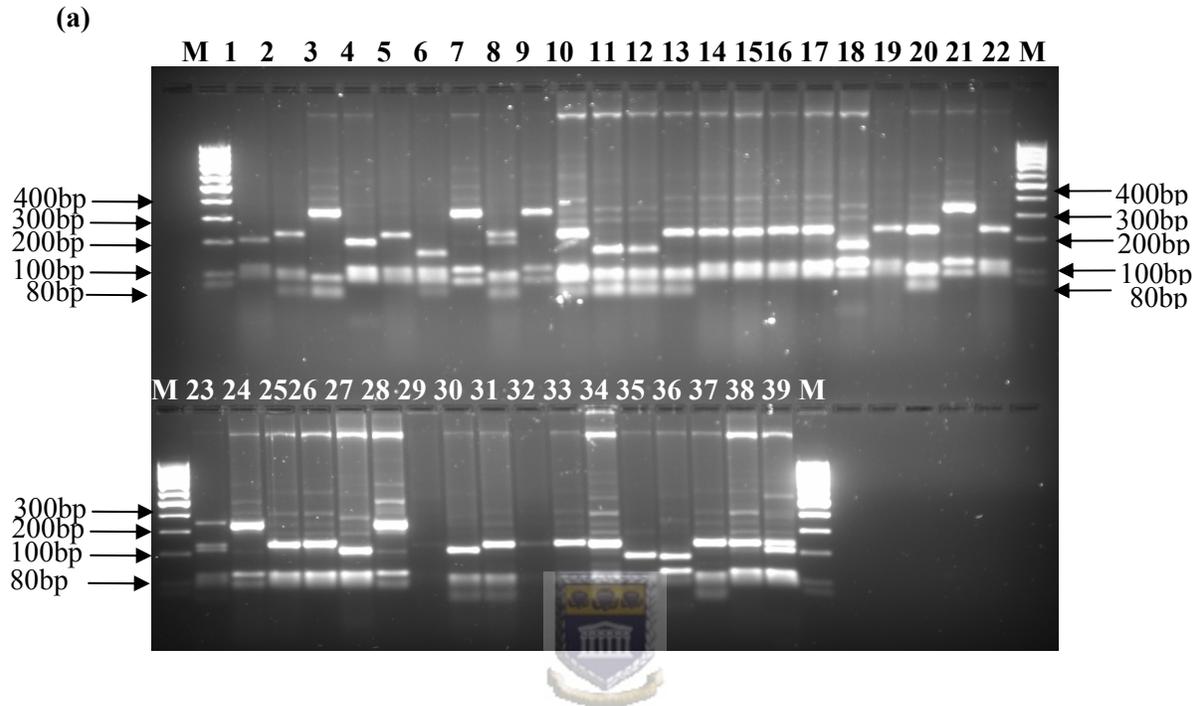
The 69 colony PCR amplicons were subjected to ARDRA in order to assess the variety of DNA sequences in the library. Among the three restriction enzymes used in this study, *Mva*I provided more detailed banding patterns than *Hpy*F10VI and *Sdu*I. Fig. 3.11 and Table 3.6 show that the *Mva*I restriction endonuclease digestion gave the best resolution yielding 11 different patterns, in comparison to *Hpy*F10VI and *Sdu*I which gave 6 and 9 different patterns, respectively. Digestion pattern A was dominant in the lower core sample (17%) while pattern C was dominant in the upper core sample (32%). Using *Mva*I restriction endonuclease digestion patterns B, D, E, G, I, J and K only occurred in the lower core sample (Table 3.6). In lane 4, for

example, the *MvaI* digestion of PCR produced fragment (514 bp) 220, 100, 120 and 70 bp fragments, in this case the DNA, however the 70 bp fragment was very faint (lanes 1, 4) Digestion pattern in lane 2, producing 230, 100, 50 and 40 bp fragments after digestion, was found to be different from the one in lane 3 and also in lane 4. Patterns in lane 3 and 7, with 330, 100 and 70 bp fragments, were also found to be different to that of lane 6, with 180, 100, 90, 80 and 60 fragments as well as to that in lane 10, with 210, 100, 80, 80 and 40 bp fragments.

Digestion pattern A was dominant in both samples for *HpyF10VI* and *SduI* (39% and 25%, respectively), and pattern C was dominant in both samples (51%), and E (17%) was only dominant in upper core sample for *HpyF10VI* and *SduI*, respectively (Table 3.7 b and c). The PCR fragment (514 bp) produced 330, 100, and 80 bp fragments after *HpyF10VI* digestion and 260, 100 and 50 bp fragments, respectively, (lanes 1, 6 and 7) and (lanes 2, 4 and 5). All fragment sizes added up to the expected size (514 bp). For some unknown reason, digestion patterns in lane 3 and 28 gave multiple bands that did not add up to the expected size (Fig. 3.11 b). Digestion pattern D only occurred in lower core sample and pattern E and F occurred in upper core sample (Table 3.7 b).

The *SduI* digestion of PCR fragment (514 bp) produced 250, 220, and 60 bp fragments and 250, 180, and 100 fragments (lanes 1, 2, 5, 7 and 8) and lane 3, respectively. DNA was not digested, presumably because no *SduI* site was present

(lanes 26) (Fig. 3.11 c). Digestion patterns D, and G only occurred in lower core sample and pattern H and I only in upper core sample (Table 3.7 c).



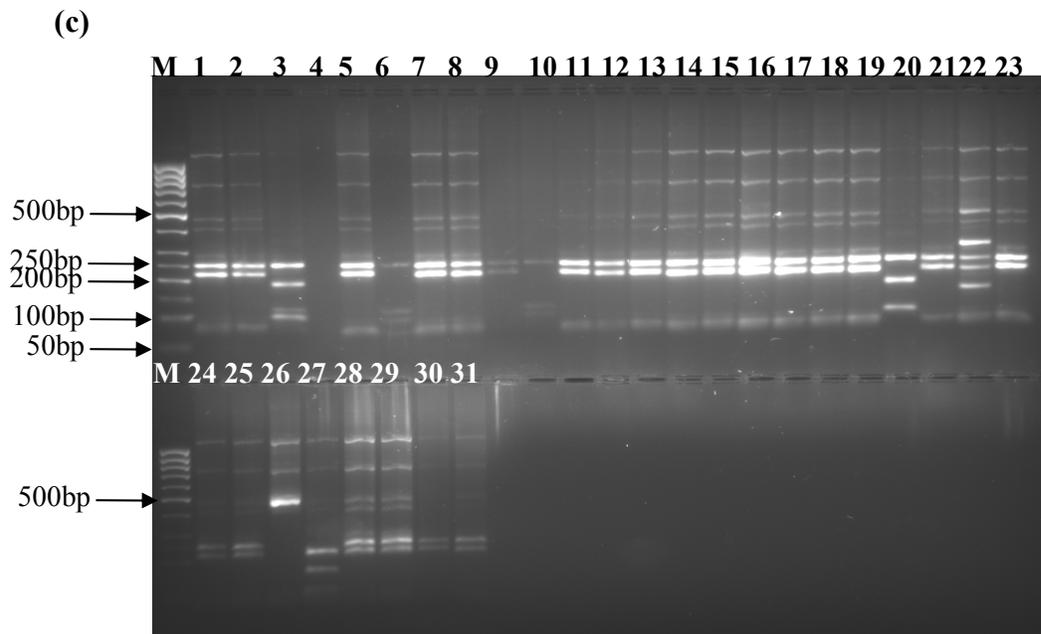


Fig. 3.11. ARDRA patterns of 16S rDNA PCR products of *Syntrophobacterales* using pUCM13F and pUCM13R primers. **a-c:** Representative of DNA of colonies from lower core and upper core sample, digested by *MvaI*, *HpyF10VI* and *SduI* restriction endonuclease, respectively, separated on a 2.5% agarose gel. Lanes: M and M₁, DNA ladders (Fermentas SM 1108; SM 1138, respectively); 1-39, PCR products obtained from white colonies that were chosen randomly from lower core and upper core samples.

From each unique ARDRA pattern, a representative clone was sequenced (Table 3.6).

Table 3.7 a. Analysis of ARDRA patterns from 16S rRNA genes using *MvaI* restriction endonuclease digestion

Pattern	Clones from Lower core sample	Clones from Upper core sample
A	B1, B4, B16, B17, B18, B19, B21, B25, B28, B29, B43, B44	T44
B	B2, B5	T4, T5, T8, T14, T41, T45
C	B3, B27, B31, B35, B37, B38, B42	T2, T3, T11, T12, T13, T15, T16, T17, T19, T21, T22, T24, T26, T27, T28, T30, T32, T35, T39, T42, T43, T46
D	B6, B20, B30	N/A
E	B7, B10, B23	N/A
F	B8	N/A
G	B11, B15, B22	N/A
H	B13, B14, B34, B39	T18
I	B26	N/A
J	B33, B36	N/A
K	B40	N/A

N/A not applicable

B clones that were selected from lower core sample

T clones that were selected from upper core sample



Table 3. 7 b. Analysis of ARDRA patterns from bacterial 16S rRNA genes using *HpyF10VI* restriction endonuclease digestion

Pattern	Clones from Lower core sample	Clones from Upper core sample
A	B1, B2, B3, B4, B6	T2, T4
B	B5, B7, B10, B11, B18, B19, B20, B21, B23, B25, B26, B31, B34, B35, B36, B42, B43, B40	T5, T8, T11, T16, T18, T22, T24, T28, T42
C	B8, B26, B44	T21, T30, T44
D	B13, B14, B16, B17, B22, B30, B38, B39	N/A
E	N/A	T3, T12, T14, T15, T17, T19, T26, T32, T34, T39, T41, T43
F	N/A	T45

N/A not applicable

B clones that were selected from lower core sample

T clones that were selected from upper core sample

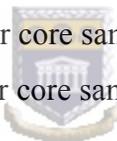
Table 3.7 c. Analysis of ARDRA patterns from 16S rRNA genes using *SduI* restriction endonuclease digestion

Pattern	Clones from Lower core sample	Clones from Upper core sample
A	B1, B2, B4, B6, B11, B13, B14, B16, B17, B30, B34, B37, B38, B42, B44	T1, T15
B	B3, B15, B25, B35, B43	T4, T28, T43
C	B5, B7, B8, B10, B18, B19, B20, B21, B22, B27, B28, B29, B39, B40	T2, T3, T5, T13, T14, T16, T17, T18, T19, T21, T22, T24, T26, T27, T30, T34, T39, T41, T44, T45, T46
D	B23	N/A
E	B26, B31	T42
G	B33, B35	N/A
H	N/A	T5
I	N/A	T30

N/A not applicable

B clones that were selected from lower core sample

T clones that were selected from upper core sample



3.3.4. *Syntrophobacterales* 16S rDNA sequence analysis

Prior to BLAST analysis, sequences of vector and primers were excluded, leaving just the insert to be analysed. Sequence lengths ranged from 491 to 501 nucleotides. All of the sequences retrieved fell within the phylum *Proteobacteria*. Half of the clones (49%) showed high homology to cultured species. The remaining clones (52%) were relatively close to uncultured species and therefore could not be classified at the species level (Table 3.8). However, partial sequencing followed by BLAST analyses demonstrated that only 20 clones from the library belonged to *δ-proteobacteria*, and 9 clones belonged to *α-proteobacteria* (Fig. 3.12). Approximately 28% of sequences exhibited more than a 5% divergence from

sequences in GenBank database. Sequences of clones with <95% identity to those in the NCBI database suggest that these clones belonged to different and possibly novel genera.

The neighbour-joining phylogenetic tree (Fig. 3.12) included the twelve families of the *Proteobacteria*. The micrococcus *Arthrobacter globiformis* was used to root the tree. From the 29 clones, six and one of the clones belonged to *Syntrophaceae* and *Syntrophobacteraceae* families, respectively, eight to an unknown family. Three of the clones belonged to *Haliangiaceae*, one to *Myxococcaceae*, one to *Desulfobacteraceae*, and the remaining eight were distributed within the families of α -*Proteobacteria* (Fig. 3.12).



Clones B40 and B20 placed within the family *Syntrophaceae* affiliated with uncultured *Syntrophus* sp. with bootstrap value of 87% and were closely related to *Syntrophus* sp. Clone T18 was closely related to *Syntrophus gentianae* and was affiliated with *Syntrophus buswellii* and uncultured *Syntrophus* sp. with a bootstrap value of 90%. Clones B4 and B6 were affiliated with uncultured *Syntrophus* sp. Clone T21 affiliated with sulfate reducing bacterium with a bootstrap value of 92%. The clones, T5, T8, B5, T41, and T45 branched with the already known sulfate reducing bacterium, but were not assigned with any known families. Clone B42 was closely related to δ -*proteobacterium* and its branch was affiliated to B11 with a bootstrap value of 99%. These clones might represent a new order, and it is reasonable to assume that these clones represent a novel lineage at taxonomic level.

Clone B15 affiliated with *Desulfosarcina* sp., with a bootstrap value of 93% and was closely related to *Desulfococcus multivorans*. Clone T27 was closely related to *Anaeromyxobacter dehalogenans*. Clone B25 placed within the *Syntrophobacteraceae*, was closely related to *Syntrophobacter pfennigii* which affiliated with *Syntrophobacter wolinii* with a bootstrap value of 99%. Clone B3, B13 and B17 were closely related to *Haliangium tepidum*.



Table 3.8. Blast analysis of 16S unique rDNA insert sequences

Clone	% Identity	Nearest Organism match	Accession Number
B2	96	Uncultured <i>Syntrophus</i> sp.	AY261813
B3	96	Uncultured δ - proteobacterium	AY499729
B4	95	Uncultured <i>Syntrophus</i> sp.	AY780562
B5	98	Uncultured sulfate-reducing bacterium	AB069773
B6	95	<i>Syntrophus</i> sp.	AJ133794
B7	99	<i>Labrys</i> sp. CC-BB4	DQ062742
B11	93	Uncultured δ - proteobacterium	AY940124
B13	95	Uncultured δ - proteobacterium	AM071378
B15	99	Uncultured δ - proteobacterium	AB074950
B17	90	<i>Pelobacter carbinolicus</i> DSM 2380	CP000142
B20	97	<i>Syntrophus</i> sp.	AJ133794
B23	97	<i>Sphingomonas</i> sp.	AJ011505
B25	96	Uncultured <i>Syntrophobacteraceae</i> bacterium	AY167444
B31	99	Uncultured α -proteobacterium clone	AY921929
B40	98	<i>Smithella propionica</i>	AF126282
B42	98	δ - proteobacterium UI	AB212873
T2	97	<i>Rhodobacter</i> sp. R-8	AY914074
T3	96	<i>Sinorhizobium</i> sp. c37	AB167207
T5	98	Uncultured sulfate-reducing bacterium	AB069774
T8	98	Uncultured sulfate-reducing bacterium	AB069774
T15	90	<i>Stella humosa</i>	AJ535710
T18	99	<i>Syntrophus gentianae</i>	X85132
T21	88	Uncultured sulfate-reducing bacterium	AB069773
T27	91	Uncultured δ - proteobacterium	AY164375
T28	96	<i>Catellibacterium nectariphilum</i>	AB101543
T30	98	<i>Sphingomonas</i> sp. CS101	AY522503
T41	98	Uncultured sulfate-reducing bacterium	AB069773
T42	98	<i>Roseomonas lacus</i>	AJ78600
T45	98	Uncultured sulfate-reducing bacterium	AB069773

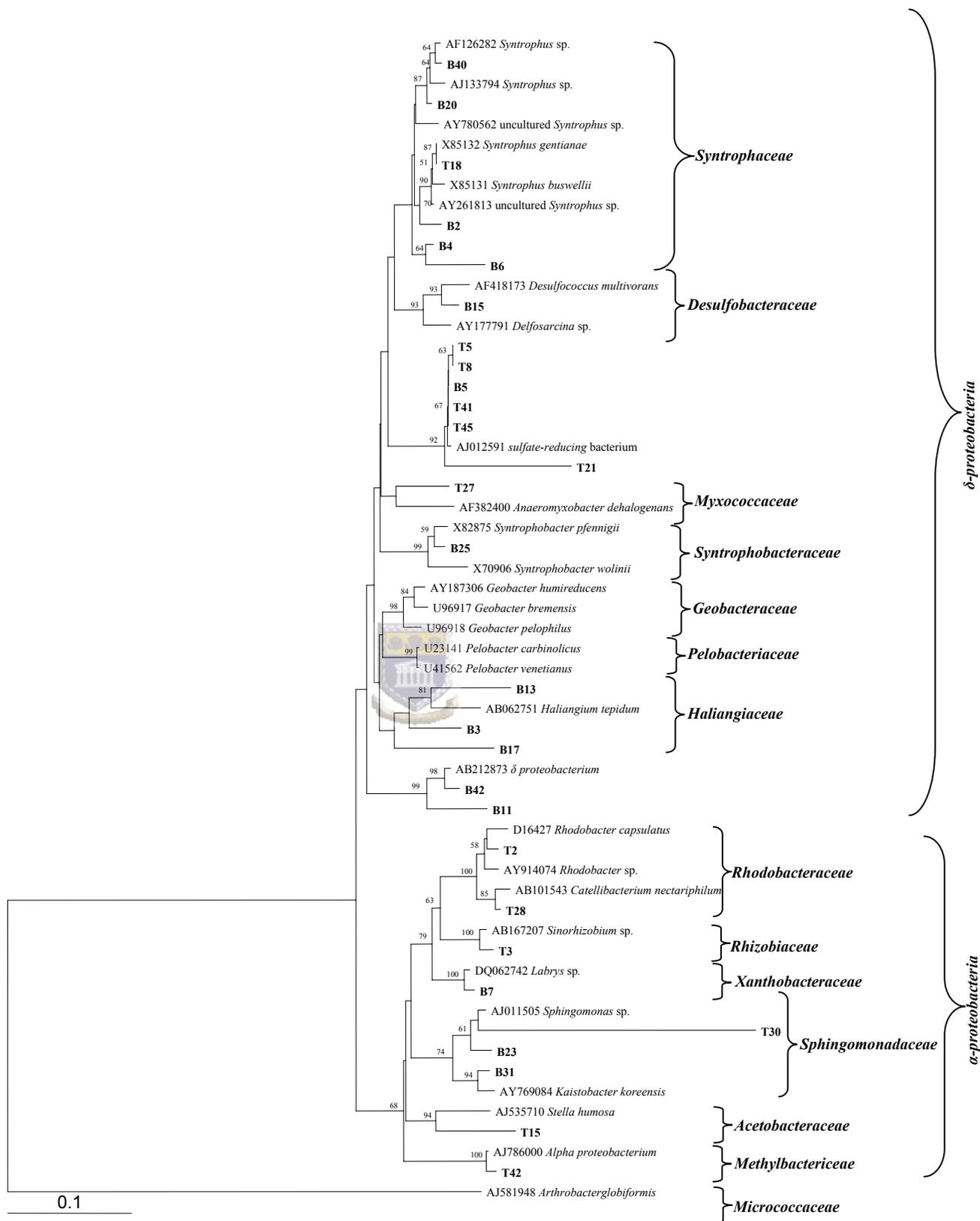


Fig. 3.12. Phylogenetic tree of partial 16S rRNA gene sequences from domain *Bacteria*, constructed with Phylo_win 2.0 using the neighbour-joining algorithm. Bar = 10% estimated sequence divergence. The sequences obtained in the present study are shown in bold letters.

Numbers at nodes represent the percentages of bootstrap resamplings based on 1000 replicates; only values of >50 are represented. *Arthrobacter globiformis* was used as an outgroup.



Chapter 4: Discussion

4.1 Analysis of the methanogenic community in the wetland

Methanogenesis can be viewed as the end process of a complex series of trophic interactions in which several groups of bacteria work together to oxidise organic carbon, leading to the production of methane (Chauhan *et al.*, 2004). The degradation of organic matter among methanogenic microbial communities is an ongoing process, and is critical since metabolic products of some microorganisms serve as substrates for other organisms (Kotsyurbenko, 2005).

Trophic interactions within such communities are determined by the thermodynamics of respective biochemical reactions. The structural polymers that occur in anaerobic zones of various ecosystems are degraded by hydrolytic microorganisms with the production of monomeric compounds, particularly carbohydrates. The latter serve as substrates for primary fermentative anaerobes, which produce hydrogen and various volatile fatty acids. The fatty acids are further utilized by syntrophic bacteria with the formation of acetate and hydrogen. H_2 , together with environmental CO_2 , is assimilated by the archaeal partners, the methanogen, to form methane (Moreira and García, 1998).

In this study, a syntrophic-methanogenic consortium was characterized at the primary inflow of the wetland of the Middelvlei winery in Stellenbosch. The consortium was analysed in order to confirm that samples that were collected from winery wetland contain organisms that process organic compounds such as acetate that are important

in the biosolubilisation of coal. DNA was extracted using the Miller method and this method gave a high molecular mass with little evidence of shearing. As it was initially difficult to extract DNA from these samples, the influence of soil pre-treatment and storage conditions was investigated. Methods for extraction and purification of DNA from soil were optimised. Optimisation of DNA extraction methods included comparisons using FastDNA spin Kit (Bio 101), Zhou, and Miller methods. It was observed that there was no significant difference between samples that had been frozen and those that were collected and extracted immediately. However, the samples that were extracted using the vortex method from the Miller protocol produced high molecular weight DNA. The FastPrep protocol produced DNA that was sheared extensively, irrespective of time and method.



The wetland methanogenic community was investigated by generation of a clone library and Amplified rDNA Restriction Analysis. The quality of DNA extracted from wetland soil was adequate for PCR. Products obtained from the M30F and M707R primer set had the expected size of 367 bp, with clear distinctive bands when viewed on the agarose gel. ARDRA analysis showed more unique patterns within the lower core sample, in comparison to the upper core sample. This is expected as methanogens are strict anaerobes and extremely oxygen sensitive. PCR amplification of 16S rDNA, and subsequent sequencing of clones combined with DGGE, was used to obtain information on the variability of the archaeal communities in the wetland. From the twenty two (22) clones that were analysed, the following conclusions have been made. The consortium was dominated by *Methanomicrobiales* species (52%),

which are considered to be hydrogenotrophic, and *Methanosarcinales* species (38%), which are acetotrophic. Very few *Methanobacteriales* were present. *Methanosarcinales* typically thrive in low acetate concentrations, as has been reported for most previous studies of unmodified wetlands (Utsumi *et al.*, 2003; Galand *et al.*, 2003; and Basiliko *et al.*, 2003). It was expected to find *Methanosarcinales* because samples were collected during non-harvesting season (November) and during that time the winery effluents are known to contain a low concentration of acetate.

Most of the sequences obtained showed high homology to the sequences of uncultured *Methanomicrobiaceae* and uncultured *Methanosaetaceae* species. Our results demonstrated the potential for both hydrogenotrophic and acetoclastic methanogenesis at the studied site, for example, *Methanobacterium formicicum* and *Methanosaeta concilii*. Data in this study support recent studies such as those conducted by Hoj *et al.* (2005) and Chauhan *et al.* (2004). Members of *Methanomicrobiales* and *Methanobacteriaceae* grow exclusively by a CO₂-reduction pathway, using one or more of the substrates H₂/CO₂, formate and short-chained alcohols. Known members of the *Methanosaetaceae* grow exclusively using acetate as the energy source (Hoj *et al.*, 2005). Kotsyurbenko *et al.* (2004) showed the occurrence of such members only in environments where most of the organic matter was made up of polysaccharides (e.g., lake sediments and rice paddy soil).

Clones T2 and T15 were closely related to a methanogenic endosymbiont of *Caenomorpha*- like sp. 1 and methanogenic endosymbiont of *Caenomorpha*- like sp. 2, respectively. *Caenomorpha* is a free-living anaerobic heterotrichous ciliated protozoan that is adapted to an anaerobic life style (van Hoek *et al.*, 2000). This protozoan has evolved hydrogenosomes, which arose from mitochondria in the course of their adaptation to anaerobic environments. Hydrogenosomes produce ATP and hydrogen and hydrogenosome-bearing ciliates acquired methanogenic endosymbionts that use intracellular hydrogen for the reduction of CO₂. Such an endosymbiont association is obviously mutualistic, since the functioning of the hydrogenosomes requires a low partial pressure of hydrogen that can be guaranteed by methanogenic archaea that use intracellular hydrogen as a substrate for methane formation (van Hoek *et al.*, 2000). This suggests that *Caenomorpha*- like sp. might occur in the wetland, a proposal supported by Schink (1997) who showed that anaerobic protozoan can be associated with symbiotic methanogens, either extracellularly or intracellularly. Ciliates living in strict anoxic, eutrophic sediments carry methanogenic partner bacteria inside the cell. Removal of hydrogen and maintenance of a low hydrogen and formate concentration in the cell allow the fermentation of complex organic matter mainly acetate and CO₂.

Although most sequences shared homology to *Methanomicrobiales* and *Methanobacteriales* or *Methanosarcinales*, no sequences belonging to the order *Methanococcales* were detected in the wetland. This could have been due to the primer bias set during PCR amplification, rather than true absences within the

environment. The primer pair M340F and M707R that was used for PCR was designed by Watanabe *et al.*, 2004. In his study, he stated that the primer pair was modified from PARCH 340f (used for identifying *Archaea* for DGGE analysis) and 069aR, after aligning them with 77 sequences from the database and detailed BLAST analysis. The sequences used for alignment were those belonging to the following orders *Methanomicrobiales*; *Methanobacteriales*, *Methanococcales*, *Methanosarcinales*. The sequences used for alignment within the order *Methanococcales* included *Methanococcus jannaschii* (M51926). After failure to detect any members of the *Methanococcales*, the 16S rDNA sequences of *Methanococcus jannaschii* (M51926) and *Methanosarcina barkeri* (AF028692) were retrieved from the NCBI database in order to validate Watanabe's primer set (Fig. 4.1). Fig. 4.1 shows that the forward primer (M340F) was targeted (indicated with asterisks) within both sequences but that the reverse primer was not targeted fully homologous to the *Methanococcus jannaschii* sequence, which lacked six bases. From this observation, it can be suggested that this primer set could be useful in targeting orders among *Euryarchaeota*, with the exception of *Methanococcales* so other primers that are inclusive to this order should be used for future applications.

M51926 <i>Methanococcus jannaschii</i>	320	*	*	345...
AF028692 <i>Methanosarcina barkeri</i>	TCCAGG	CCCTACGGGGCGCAGCAG	GC	
Forward primer (M340F)	GG	CCCTACGGGGCGCAGCAG	GCGCGA.....	
		CCCTACGGGGCGCAGCAG		
M51926 <i>Methanococcus jannaschii</i>	610		*	* 654
AF028692 <i>Methanosarcina barkeri</i>	CCNGGGGTACCCCAGGGGTAGCG	GTGAAATGCGT	TGATCCCTGG	GGGA
Reverse primer (M707R)	AAGAGGTACTACAGGGGTAGGA	GTGAAATCTTGTAAATCCCTGT	.GGGGA	
		GTGAAATCTTGTAAATCCCTGT		

Fig. 4.1. Alignment showing mismatches of the *Methanococcus jannaschii* rDNA with the reverse primer (M707R)

Primer set M340F and M707R has the potential to amplify 16S rDNA of non-methanogenic archaea, for example, clone T26 which was found to contain bacterial 16S rDNA.

4.2 Analysis of the syntrophic community in the wetland

Hydrogen-utilising fatty acid oxidizing bacteria, frequently referred to as syntrophs, are secondary fermenters that work together with methanogens to oxidise primary fermentation products such as propionate and butyrate that cannot be utilized directly by methanogens. These organisms play an important role in decomposition of organic matter under methanogenic conditions (Chauhan *et al.*, 2004). One of the major modes of bacterial symbiosis involves the exchange of H₂ between different organisms. This symbiosis occurs extensively between archaea and bacteria, and is metabolically advantageous for both partners (Moreira and García, 1998).

A further aim of this study was to identify syntrophic microorganisms in the wetland system. In order to achieve this goal, primers Syn682F and Syn1196R were designed. The primers were designed to target members of *Syntrophobacterales*, in particular members of the *Syntrophaceae* and *Syntrophobacteraceae* families. To the best of our

knowledge, no primer sets specific to these two families have been reported. To test the primer specificity gradient PCR was performed to determine the highest possible annealing temperature suitable for effective PCR.

In order to determine the most suitable annealing temperature, PCR trials were performed over a temperature range of 52 °C to 62 °C. It was noted that the *E. coli* control was amplified even at 62 °C. A second PCR trial was performed over a broader range of temperature, from 55 °C to 72 °C. The results demonstrated that an annealing temperature between 64.2 °C and 65 °C was ideal for this primer set since no *E. coli* products were targeted. The PCR products obtained had the expected size of 514 bp.



Clone libraries were subjected to ARDRA with the restriction enzymes *MvaI*, *HpyF10VI*, and *SduI*. Representatives of twenty nine (29) unique restriction pattern were sequenced for phylogenetic analysis. 20 clones from the constructed 16S rDNA library belonged to δ -*proteobacteria*, and 9 clones belonged to α -*proteobacteria*.

Many of the syntrophic phylotype signals found in the wetland samples were homologous to uncultured phylotypes, while others were closely related to *Syntrophobacter pfenning*, *Syntrophus gentianae*, *Syntrophus* sp. *Smithella propionica* and *Pelobacter carbinolicus*. These syntrophs use a variety of substrates (Schink, 1997). For example *Pelobacter* spp. are ethanol and 2, 3- butanediol oxidising syntrophs. Syntrophically propionate-degrading bacteria produce acetate,

H₂ and formate. Syntrophs require low H₂ concentrations to ferment these substrates to CO₂, and hydrogenotrophic methanogens are responsible for maintaining appropriate concentrations (Chauhan *et al.*, 2004). *Syntrophus* sp. degrades aromatics such as benzoate and gentisate (Schink, 1997). These statements further suggest these substrates from syntrophs will apparently be available for methanogens. The results confirm the presence of syntrophs but with low diversity. However, a primer pair targeted only a few genera of syntrophs and hindered homology in 16S gene does not allow more specific primer sets to be designed and cannot be improved. For future analyses, other members of *Firmicutes* could be included. This would improve our knowledge about the substrates present in the wetland. For example, genera of *Syntrophospora* and *Syntrophomonas* are known to oxidise butyrate and longer fatty acids. Members of the genus *Clostridium* are also known to oxidise acetate whereas *Syntrophobotulus* oxidise glycolate. These investigations would require the design of several novel primer sets, targeting all these genera.

In conclusion, the identification of microbial phylotypes, some of which might represent novel taxonomic lineages, suggests a well established syntrophic relationship within the wetland microbial community. However, to fully understand the syntrophic relationships which exist in this site, further experiments investigating correlations between the presence of microorganisms with their function should be conducted.

References

- Ahring, B.K.** (2003) Perspectives for anaerobic digestion, *In*: T. Scheper (ed.), *Biomethanation I*. Springer-Verlag GmbH, Heidelberg. pp. 2-30.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J.** (1990) Basic local sequence alignment search tool. *Journal of Molecular Biology* **215**: 403-410.
- Angenent, L.T., Karim, K., Al-Dahhan, M.L., Wrenn, B.A., and Domínguez-Espinosa, R.** (2004) Production of bioenergy and biochemicals from industrial and agricultural wastewater. *TRENDS in Biotechnology* **9**: 477-485.
- Atlas, R.M., and Bartha, R.** (1998) *Microbial ecology fundamentals and applications*. Fourth edition. Cummings Science Publishing, London. pp. 69-75.
- Baker, G.C., Smith, J.J., and Cowan, D.A.** (2003) Review and re-analysis of domain-specific 16S primers. *Journal of Microbiological Methods* **55**: 541-555.
- Barik, S., Isbister, J., Harding, R., and Berrill, P.** (1991) Coal biogasification research and process development. *In*: Yunjer, S. (ed.), Proceedings of the Second International Symposium on the Biological Processing of Coal. Electric Power Research Institute, Palo Alto, Calif. Section **6**: 23-38.
- Basiliko, N., Yavitt, J.B., Dees, P.M., and Merkrl, S.M.** (2003) Methane biogeochemistry and methanogen communities in two northern peat land ecosystems, New York State. *Geomicrobiology Journal* **20**: 563-577.
- Berry, D.F., Francis, A.J., and Bollag, J.M.** (1987) Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions. *Microbiological Reviews* **51**: 43-59.

- Berthelet, M., Whyte, L.G., and Greer, C.W.** (1996) Rapid, direct extraction of DNA from soils for PCR analysis using polyvinylpolypyrrolidone spin columns. *FEMS Microbiology Letters* **138**: 17-22.
- Budwill, K.** (2003) Microbial methanogenesis and its role in enhancing coalbed methane recovery. *CSEG Recorder* **41**: 41-46.
- Cairncross, B.** (2001) An overview of the permian (karoo) coal deposits in Southern Africa. *Journal of African Earth Sciences* **33**: 529-562.
- Catcheside, D.E.A., and Ralph, J.P.** (1999) Biological processing of coal. *Applied Microbiology and Biotechnology* **52**: 16-24.
- Chauhan, A., Ogram, A., and Reddy, K.R.** (2004) Syntrophic-methanogenic association along a nutrient gradient in the Florida everglades. *Applied and Environmental Microbiology* **70**: 3775-3484.
- Claassen, P.A.M., van Lier, J.B., Lopez Contreras, A.M., van Niel, E.W.J., Sittsma, L., Stams, A.J.M., de Vries, S.S., and Westhuis, R.A.** (1999) Utilisation of biomass for the supply of energy carriers. *Applied Microbiology and Biotechnology* **52**: 741-755.
- Cohen, M.S., and Gabriele, P.D.** (1982) Degradation of coal by the fungi *Polyporus versicolor* and *Poria monticola*. *Applied and Environmental Microbiology* **44**: 23-27.
- Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., Schmidt, T.M., Garrity, G.M., and Tiedje, J.M.** (2003) The Ribosomal database project (RDP-II): previewing a new autoligner that allows regular updates and new prokaryotic taxonomy. *Nucleic Acids Research* **31**: 442-443.

Colin, T., Bories, A., Sire, Y., and Perrin, R. (2005) Treatment and valorization of winery wastewater by a new biophysical process (ECCF). *Water Science and Technology* **51**: 99-106.

Conrad, R. (1999) Contribution of hydrogen to methane production and control of hydrogen concentration in methanogenic soils and sediments. *FEMS Microbiology Ecology* **28**: 193-202.

Conway de Macario, E., and Macario A.J.L. (2003) Molecular biology of stress in methanogens: potential for bioreactors technology, *In*: T. Scheper (ed.), Biomethanation I. Springer-Verlag GmbH, Heidelberg. pp. 95-151.

Crawford, D.L., Gupta, R.K., Deobald L.A., and Roberts, D.J. (1990) Biotransformation of coal and coal substructure model compounds by bacteria under aerobic and anaerobic conditions. *In*:  Yunker S, Rhee K (eds.), Proc First Int Symp Biological Processing of Coal. Electric Power Research Institute, Palo Alto, Calif., pp. 429-443.

Crawford, D.L., and Gupta, R.K. (1993) Microbial depolymerization of coal. *In*: Crawford DL (ed.), Microbial transformation of low rank coals. CRC, Boca Roton, Florida. pp. 65-92.

Davison, B.H., Nicklaus, D.M., Misra, A., Lewis, S.N., and Faison, D.B. (1990) Utilization of microbially solubilized coal-preliminary studies on anaerobic conversion. *Applied Microbiology and Biotechnology* **24**: 447-456.

de Bok, F.A.M., Harmsen, H.J.K., Plugge, C.M., de Vries, M.C., Akkermans, A.D.L., de Vos, W.M., and Stams, A.J.M. (2005) The first true obligatery syntrophic propionate-oxidizing bacterium, *Pelotomaculum Schinkii* sp. nov.,

co-cultured with *Methanospirillum hungatei*, and emended description of the genus *Pelotomaculum*. *International Journal of Systematic and Evolutionary Microbiology* **55**: 1697-1703.

Dunbar, J., Takala, S., Barns, S.M., Davis, J.A., and Kuske, C.R. (1999) Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. *Applied and Environmental Microbiology* **65**: 1662-1669.

Engesser, K.L., Dohms, C., and Schmid, A. (1994) Microbial degradation of model compounds of coal and production of metabolites with potential commercial value. *Fuel Processing Technology* **40**: 217-226.

Eusébio, A., Mateus, M., Baeta-Hall, L., Almeida-Vara, E., and Duarte, J.C. (2005) Microflora evaluation of two agro-industrial effluents treated by the JACTO jet-loop type reactor system. *Water Science and Technology* **51**: 107-112.

Evans, L.J., Fernandez, I.J., Rustad, L.E., and Norton, S.A. (2001) Methods for evaluating carbon fractions in forest soils: A Review. *Maine Agricultural and Forest Experiments Station Technical Bulletin* **178**: 4-42.

Faison, B.D. (1991) Biological coal conversions. *Critical Reviews in Biotechnology* **11**: 347-366.

Faison, B.D. (1993) The chemistry of low rank and its relationship to the biochemical mechanism of coal biotransformation. *In*: Crawford DL (ed.), *Microbial coals*. CRS Press. Boca Roton, Florida, pp. 1-26.

Farely, V., Rainey, F., and Stackebrandt, E. (1995) Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Applied and Environmental Microbiology* **61**: 2798-2801.

Faukoussa, R.M., and Hofrichter, M. (1999) Biotechnology and microbiology of coal degradation. *Applied Microbiology and Biotechnology* **52**: 25-40.

Faúndez, J., Arenillas, A., Rubiera, F., García, X., Gordon, A.L., and Pis, J.J. (2005) Ignition behaviour of different rank coals in an entrained flow reactor. *Fuel* xxx 1-6, *In Press*.

Galand, P.E., Fritze, H., and Yrjälä, K. (2003) Microsite dependent changes in methanogenic populations in a boreal oligotrophic fen. *Environmental Microbiology* **5**: 1133-1143.

Galtier, N.C., Galtier, M., and Gautier, C. (1996) SEAVIEV and PHYLO_WIN: two graphical tools for sequence alignment and molecular phylogeny. *CABIOS* **12**: 543-548.

Gavala, H.N., Angelidaki, I., and Ahring, B.K. (2003) Kinetics and modeling of anaerobic digestion process, *In*: T. Scheper (ed.), *Biomethanation I*. Springer-Verlag GmbH, Heidelberg. pp.58-91.

Gockay, C.F., Kolankaya, N., and Dilek, F.B. (2001) Microbial solubilisation of lignites. *Fuel* **80**: 1421-1433.

Götz, G. K., and Fakoussa, R.M. (1999) Fungal biosolubilisation of rhenish brown coal monitored by Curie-point pyrolysis/gas chromatography/mass spectrometry using tetraethylammonium hydroxide. *Applied Microbiology and Biotechnology* **52**: 41-48.

Grotenhuis, J-T.C, Smith, M., Plugge, C.M., Yuansheng, X., van Lammeren, A-A.M., Stams, A-J.M., and Zehnder, A.J.B. (1991) Bacteriological composition

and structure of granular sludge adapted to different substrates. *Applied and Environmental Microbiology* **57**: 1942-1949.

Hall, T.A. (1999) Bioedit: a userfriendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**:95-98.

Hall, B.G. (2001) *Phylogenetic Trees Made Easy: A How-to Manual for Molecular biologists* Sinauer Associates, Inc, Publishers, USA. pp. 3-26.

Hansen, M.C., Tolker-Neilson, T., Givskov, M., and Molin, S. (1998) Biased 16S rDNA PCR amplification caused by interference from DNA flanking template region. *FEMS Microbiology Ecology* **15**:25-36.

Hofman-Bang, J., Zheng, D., Westermann, P., Ahring, A., and Raskin, K. (2003) Molecular ecology of anaerobic reactor systems, *In*: T. Scheper (ed.), Biomethanation I. Springer-Verlag GmbH, Heidelberg. pp.153-198.

Hofrichter, M., Bublitz, F., and Fritsche, W. (1997) Fungal attack on coal II. Solubilization of low-rank by filamentous fungi. *Fuel Processing Technology* **52**: 55-64.

Høj, L., Rolf, R.A., and Torsvik, V.L. (2005) Archaeal communities in high arctic wetlands at Spitsbergen, Norway (78°N) as characterized by 16S rRNA gene fingerprinting. *FEMS Microbiology Ecology* **53**: 89-101.

Holker, U., Ludwig, S., Scheel, T., and Hofer, M. (1999) Mechanism of coal solubilisation by the deuteromycetes *Trichoderma atroviride* and *Fusarium oxysporum*. *Applied Microbiology and Biotechnology* **52**: 57-59.

- Jackson, C.R., Harper, J.P., Willoughby, D., Rosen, E.E., and Churchill, P.F.** (1997) A simple, efficient method for separation of humic substances and DNA from environmental samples. *Applied and Environmental Microbiology* **63**: 4993-4995.
- Johnson, E.R., Klasson, K.T., Basu, R., Volkwein, J.C., Clausen, E.C., Gaddy, J.L.** (1994) Microbial conversion of high-rank coals to methane. *Applied Biochemistry and Biotechnology* **45**: 329-338.
- Jones, J.M., Pourkashanian, M, Rena, C.D., and Williams, A.** (1999) Modelling the relationship of coal structure to char porosity. *Fuel* **78**: 1737-1744.
- Karakashev, D., Batstone, D.J., and Angelidaki, I.** (2005) Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. *Applied and Environmental Microbiology* **71**: 331-338.
- Kates, M., Kusher, D.J., and Matheson, A.T.** (1993) *The Biochemistry of Archaea (Archaeobacteria)*. Volume 26. Elsevier Science Publishers, Amsterdam. pp.41-66.
- Kotsyurbenko, O.R., Chin, K.J., Glagolev, M.V., Stubner, S., Simankova, M.V., Nozhevnikova, A.N., and Conrad, R.** (2004) Acetoclastic and hydrogenotrophic methane production and methanogenic populations in acidic West-Siberian peat bog. *Environmental Microbiology* **6**: 1159-1173.
- Kotsyurbenko, O.R.** (2005) Trophic interactions in the methanogenic microbial community of low-temperature terrestrial ecosystems. *FEMS Microbiology Ecology* **53**: 3-15.
- Kuske, C.R., Banton, L.K., Adorada, D.L., Stark, P. C., Hill, K.K., and Jackson, P.J.** (1998) Small-scale DNA sample preparation method for field PCR direction on

microbial cells and spores in soil. *Applied and Environmental Microbiology* **64**: 2463-2472.

Laborda, F., Fernández, M., Luna, N., and Monistrol, I.F. (1997) Study of the mechanisms by which microorganisms solubilise and/or liquefy Spanish coals. *Fuel Processing Technology* **52**: 95-107.

Levine, D.G., Schlosberg, R.H., and Silbernagel, B.G. (1982) Understanding the chemistry and physics of coal structure (A review). *Proceeding of the National Academics Science* **79**: 3365-3370.

Lu, L., Sahajwalla, V., Kong, C., and Harris, D. (2001) Quantitative X-ray diffraction analysis and its application to various coal. *Carbon* **39**: 1821-1833.

Madigan, M.T., Martinko, J.M., and Parker, J. (2000) Brock Biology of Microorganisms. Ninth edition. Prentice Hall International, Inc. New Jersey. Pp. 748-751.



Miller, D.N., Bryant, J.E., Madsen, E.L., and Ghiorse, W.C. (1999) Evaluation and optimisation of DNA extraction and purification procedures for soil and sediments samples. *Applied and Environmental Microbiology* **65**: 4715-4724.

Moreira, D., and López-García, P. (1998) Symbiosis between methanogenic *Archaea* and δ -*Proteobacteria* as the origin of Eukaryotes: The syntrophic hypothesis. *Journal of Molecular evolution* **47**: 517-530.

Moyer, C.L., Tjieje, J.M., Dobbs, F.C., and kaRL, D.M. (1996) A Computer-simulated restriction fragment length polymorphisms analysis of bacterial small-subunit rRNA genes: Efficacy of selected tetrameric restriction enzymes for

studies of microbial diversity in nature. *Applied and Environmental Microbiology* **62**: 2501-2507

Nealson, K.H. (1997) Sediment bacteria: Who's there, what are they doing, and what's new? *Annual Review of Earth and Planetary Sciences* **25**: 403-434.

Nicomrat, D., Dick, W.A., and Tuovinen, O.H. (2006) Assessment of the microbial community in a constructed wetland that receives acid coal mine drainage. *Microbial Ecology* **51**: 83-89.

Page, R.D. (1996) Tree View: an application to display Phylogenetic trees on personal computer. *Computer Applications in the Biosciences* **12**: 357-358.

Panow, A., Fitzgerald, J.M.P., and Mainwaring, D.E. (1997) Mechanisms of biologically-mediated methane evolution from black coal. *Fuel Processing Technology* **52**: 115-125.

Petruccioli, M., Duarte, J.C., and Federici, F. (2000) High-rate aerobic treatment of winery wastewater using bioreactors with free and immobilized activated sludge. *Journal of Bioscience and Bioengineering* **90**: 381-383.

Pind, P.E., Agelidalaki, I., Ahring, B.K., Stamatelatou, K., and Lyberatos, L. (2003) Monitoring and Control of anaerobic Reactors. *In*: T. Scheper (ed.) *Biomethanation II*. Springer-Verlag GmbH, Heidelberg. pp.137-179.

Polat, M., Polat, H., and Chander, S. (2003) Physical and chemical interactions in coal flotation. *International Journal of Mineral Processing* **72**: 199-213.

Quigley, D. R., Breckenridge, C.R., and Dugan, P.R. (1989a) Effects of multivalent cations on low-rank coal solubilities in alkaline solutions and microbial cultures. *Energy Fuels* **3**: 571-574.

- Quigley, D. R., Ward, B., Crawford, D. L., Hatcher, H.J., and Dugan, P. R.** (1989b) Evidence that microbially produced alkaline materials are involved in coal biosolubilization. *Applied Biochemistry and Biotechnology* **21**: 753-763.
- Ralph, J.P., and Catcheside, D.E.A.** (1997) Transformations of low-rank coal by *Phanerochaete chrysosporium* and other wood-rot fungi. *Fuel Processing Technology* **52**: 79-93.
- Reysenbach, A. L., and Pace, N.R.** (1995) Reliable amplification of hyperthermophilic archaeal 16S rRNA genes by the polymerase chain reaction. In: Robb FT, Place AR (ed.) *Archaea: A Laboratory Manual - Thermophiles*. Cold Spring Harbour Laboratory Press, New York, pp.101-106.
- Roose-Amsaleg, C.L., Garnier-Sillam, E., and Harry, M.** (2001) Extraction and purification of microbial DNA from soil and sediment samples. *Applied Soil Ecology* **18**: 47-60.
- 
- Sambrook, J., and Russell, D.W.** (2001) *Molecular cloning: A Laboratory Manual*. Third edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. Volume 1.
- Silva-Stenico, M.E., Vengadajellum, C.J., Burton, S.G., Harrison, S.T.L., Solomon, M., and Cowan, D.** (2004) Microbial conversion of low rank coal to high-value organic products. In: 12th International Biotechnology Symposium and Exhibition. October 17-22. Santiago, Chile.
- Schink, B.** (1997) Energetics of syntrophic cooperation in methanogenic degradation. *Microbiology and Molecular Biology Review* **61**: 262-280.

- Schulter, H.R., and Schnitzer, M.** (1993) Temperature-resolved in source pyrolysis soft ionization mass-spectrometry of soil humic acids. *Organic Geochemistry* **20**: 17-25.
- Skiadas, I.V., Gavala, H.N., Schmidt, J.E., and Ahring, B.K.** (2003) Anaerobic Granular Sludge and biofilm Reactors, *In*: T. Scheper (ed.), *Biomethanation II*. pp. 36-61.
- Stach, J.E.M., Bathe, S., Clapp, J.P., and Burns, R.G.** (2001) PCR-SSCP comparison of 16S rDNA sequence diversity in soil DNA obtained using different isolation and purification methods. *FEMS Microbiology Ecology* **36**: 139-151.
- Stams, A.J.M., Oude Elferink, S.J.W.H., and Westermann, P.** (2003) Metabolic interactions between methanogenic consortia and anaerobic respiring bacteria, *In*: T. Scheper (ed.), *Biomethanation I*. Springer-Verlag GmbH, Heidelberg. pp. 32-56.
- Somerset, V.S.** The preparation and characterization of high capacity ion exchange adsorbents by co-disposal of fly ash and acid mine drainage for their possible use in electrochemical systems for water purification. *Master's thesis*, Chemistry Department, University of the Western Cape, 2003. p.2.
- Sugamo, M., Hirano, K., and Mashimo, K.** (2003) Effects of reaction on the hydrogenolyses of cation exchanged coal. *Fuel Processing Technology* **85**: 837-848.
- Thompson, J.D., Higgins, D.G., and Gibson, T.J.** (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**:4673-4680.

- Thompson, J.P., Gibson, T.J., Plewniak, F., Jeanmougin, F., and Higns, D.G.** (1997) the ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**: 4876-4882.
- Trevan, M.D., Boffey, S., Goulding, K.H., and Stanbury, P.** (1987) *Biotechnology: The Biological Principles*. Taylor and Francis. Philadelphia. pp.547-611.
- Utsumi, M., Belova, S.E., King, G.M., and Uchiyama, H.** (2003) Phylogenetic comparison of methanogen diversity in different wetland soils. *Journal of General and Applied Microbiology* **49**: 75-83.
- van Heek, K.H.** (2000) Progress of coal science in the 20th century. *Fuel* **79**: 1-26.
- van Hoek, A.H.A.M., van Alen, T.A., Sprakel, V.S.S., Leunissen, J.A.M., Brigge, B., Vogels, G.D., and Hackstein, H.P.** (2000) Multiple acquisition of methanogenic archaeal symbionts by anaerobic ciliates. *Molecular Biology and Evolution* **17**: 251-258.
- 
- Van Krevelen, D. W.** (1993) *Coal*. Third edition. Elsevier Science Publishers, Amsterdam. 1008pp.
- Vassilev, S.V., Kitano, K., and Vassileva, C.G.** (1996) Some relationship between coal rank and chemical and mineral composition. *Fuel* **75**: 1537-1542.
- Valdez-Vazquez, I., Rios-Leal, E., Esparza-Garcia, F., Cecchi, F., Pavan, P. and Poggi-Varaldo, H.M.** (2004) A review on hydrogen production with anaerobic mixed cultures. *The First International Meeting of Environmental Biotechnology and Engineering*, CINVESTAV, México City, September 6–8, 2004.
- Vignais, P.M., Billoud, B., and Meyer, J.** (2001) Classification and phylogeny of hydrogenases. *FEMS Microbiology Reviews* **25**: 455-501.

- Volkwein, J.C., Schoenman, A.L., Clausen, E.G., Gaddy, J.L., Johnson, E.R., Basu, R., Ju, N., and Klasson, K.T.** (1994) Biological production of methane from bituminous coal. *Fuel Processing Technology* **40**: 339-345.
- Watanabe, T., Asakawa, S., Nakamura, A., Nagaoka, K., and Kimura, M.** (2004) DGGE methods for analyzing 16S rDNA of methanogenic archaeal community in paddy field soil. *FEMS Microbiology Letters* **232**: 153-163.
- Wicks, R.** Coal magazine, 'The future role of coal', First Issue, Feb 2004 Available: <http://www.wci-coal.com/uploads/coalmagazine.pdf>.
- White, D.** (1999) *The Physiology and Biochemistry of Prokaryotes*. 2nd ed. University Press. Oxford. pp. 1-11.
- Wilkins, R.W.T., and George, S.C.** (2002) Coal as a source rock for oil: a review. *Coal Geology* **50**: 317-361.
- Wu, W.-M., Jain, M.K., De Macario, E.C., Thiele, J.H., and Zeikus, J.G.** (1992) Microbial composition and characterization of prevalent methanogens and acetogens isolated from syntrophic methanogenic granules. *Applied Microbiology and Biotechnology* **38**: 282-290.
- Yanga, Y., Zhang, Z., Luc, J., and Makawab, T.** (2004) Continuous methane fermentation and the production of Vitamin B₁₂ in a fixed-bed reactor packed with loofah. *Bioresource Technology* **92**:285-290.
- Ziegler, A., and Van Heek, K.H.** (1998) 9th international conference on coal science, 7-12 September 1997, Essen, Germany. *Fuel* **77**: 219-222.
- Zhou, J., Bruns, M. A., and Tiedje. J. M.** (1996) DNA recovery from soils of diverse composition. *Applied and Environmental Microbiology* **62**: 316-22.