Exploring carbon cycling in selected micro-organisms exposed to terrestrial carbon sequestration

By

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I hereby dedicate this dissertation to my family for their many years of support

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DECLARATION

I hereby declare that this thesis is submitted by me for the Magister Scientiae degree at the University of the Free State. This work is solely my own and has not been previously submitted by me at any other University or Faculty, and the other sources of information used have been acknowledged. I further grant copyright of this thesis in favour of the University of the Free State.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
˚C	Degrees Celsius
16SrRNA	Small Subunit Ribosomal Ribose Nucleic Acid
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
BSA	Bovine Serum Albumin
CS	Carbon sequestration
CCS	Carbon capture and storage
Cells/mL	Cells per millilitre
CO_2	Carbon dioxide
DNA	Deoxyribonucleic Acid
DSMZ	Deutsche Sammiung von Mikroorganismen und Zelkulturen GmbH
E. coli	*Escherichia coli*
EDTA	Ethylene Diaminetetraacetic Acid
EtBr	Ethidium bromide
EOR	Enriched oil recovery
GAP	Glyceraldehydes 3-phosphate

g/L	Gram per litre
gDNA	Genomic DNA
g	Gram
H_2	Hydrogen
HCl	Hydrochloric acid
IEA	International Energy Agency
IPCC	Intergovernmental Panel on Climate Change
IPTG	Isopropyl β-D-1-Thiogalactopyranoside
LB	Luria-Bertani
µL	Microliter
µm	Micrometre
µM	Micromolars
µmol	Micromole
min	Minute
mg/mL	Milligram per millilitre
mL	Millilitre
mM	Millimolars
mol%	Mole percentage
MOPS	3-(N-morpholino) Propanesulfonic Acid Hemisodium Salt

N_2	Nitrogen
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
ND	Nanodrop
ng/µL	Nanogram per microliter
nm	Nanometre
OD	Optical Density
PRK	Rubisco, phosphoribulokinase
PCR	Polymerase Chain Reaction
ppm	Parts per million
rpm	Revolutions per minute
rAcCOA	Reductive acetyl co-enzyme A cycle
rPP	Calvin cycle
rTCA	Reductive tricarboxylic acid cycle
RNA	Ribonucleic acid
SBPase	Sedoheptulose bisphosphatase
SC-CO_2	Supercritical CO_2
TEA	Triethanolamine
t	Tonnes

Tfb	Transformation buffer
TYG	Tryptone, Yeast extract, Glucose
UV	Ultraviolet
UV-vis	Ultraviolet-visible
U	Units
V	Volts
v/v	Volume per volume
X-gal	5-Bromo-4-Chloro-3-Indolyl-beta-D-Galactopyranosidehosphate

CHAPTER 1

CHAPTER 1

LITERATURE REVIEW

1. Introduction

Global warming is described as the rise in the average temperature of the Earth's atmosphere causing climate change. Warming of the climate system is primarily caused by increasing concentrations of greenhouse gasses such as carbon dioxide (CO_2), produced by human activities (Vitousek, 1994; IPCC, 2013). CO_2 is one of the many greenhouse gasses being emitted into the air from both natural sources and human activity. As the layer of greenhouse gasses around our planet grows thicker, more heat is trapped in the atmosphere and the Earth slowly heats up. Other contributors to the greenhouse effect are water vapour, which is the gas phase of water, methane, nitrous oxide, ozone [or triatomic oxygen (O_3)] and several other gasses that are present in the atmosphere in small amounts (Sulzman, 2000; Ledley *et al*., 2002; Wallington *et al*., 2004; IPCC, 2013).

Burning of natural gasses like coal in power plants, gasoline in cars and the activities of large industrial facilities, contribute to the level of CO_2 and related gasses in the atmosphere. In the last five to six decades, the CO_2 concentration in the Earth's atmosphere has increased vastly and will become worse in the future as human activities permit for more fossil fuels to be burnt (Marland & Boden, 2001; Ehlig-Economides & Economides, 2010). Natural activities such as volcanic eruptions, natural release of greenhouse gasses (e.g. methane) from permafrost (also known

as cryotic soil) as well as fires further contribute to the warming of the planet (Ledley *et al*., 2002; Kharaka *et al*., 2009).

To limit emission of CO_2, which results in its accumulation in the earth's atmosphere, carbon resources have to be managed more effectively. Carbon dioxide, released from power stations, fossil fuels and other related sources, can be transported and stored in deep surfaces where it is secured; a process known as carbon capture and storage (CCS) (Benson *et al*., 2008). Countries, such as South Africa, the United Kingdom, United States of America, India and China generate most of its electricity from coal. The International Energy Agency (IEA) has predicted a possible 70% global increase of coal usage in the next 20 years. Meeting these demands will increase greenhouse gasses being released into the atmosphere. As a result, capture and storage of CO_2 proves to be a very efficient process to eliminate the negative contribution towards climate change (CO_2 capture, transport and storage, 2009; Finkenrath *et al*., 2012).

Capturing and storing CO_2 may present more time for scientists to develop low-carbon technologies. This task of capturing CO_2 is still relatively new. There is limited information regarding geological CO_2 storage. Therefore, if CCS is proven to be viable technically and commercially, other applications that emit CO_2 will have to comply with the ability to retrofit CCS (CO_2 capture, transport and storage, 2009; Sherwood Lollar & Ballentine, 2009).

1.1 Climate change

The sun radiates photons of frequencies that can pass through the Earth's atmosphere, with much of its heat energy in the infrared band. Warming of the lowering atmosphere is due to the changes that occur with the infrared energy, such as absorption and re-radiation by the earth's greenhouse gasses. Indeed, natural greenhouse gasses over the Earth's history have made life more comfortable for humans to live in, but the dramatic effects of anthropogenic (man-made) CO_2 have led to a further rise in global temperature, which leads to heat stress causing fatalities from natural phenomena (Wallington *et al*., 2004; IPCC, 2007; Sherwood & Huber, 2010).

Since 1958, atmospheric CO_2 levels has been monitored and records now indicate that CO_2 levels have risen with 390.5 ppm (parts per million) in 2011; from an average of 316.0 ppm in 1959, shown in figure 1.1 (Keeling, 1960; IPCC, 2001; Ledley *et al*., 2002; IPCC, 2007; Velea *et al*., 2009; IPCC, 2013). The Intergovernmental Panel on Climate Change (IPCC, 2001) has made certain predictions regarding rising levels of CO_2. These include environmental effects when temperature increases which have negative impacts on livestock and wildlife, for example, heat stress on humans and related species (Nye *et al*., 2007; Sherwood & Huber, 2010). Fossil fuels will be the dominant energy source for future energy requirements, as the future usage of fossil fuels will determine the rise in atmospheric CO_2 levels associated with global warming, which will increase 1.1 to 6.4˚C to the present temperature in 2100 (NRC, 2010).

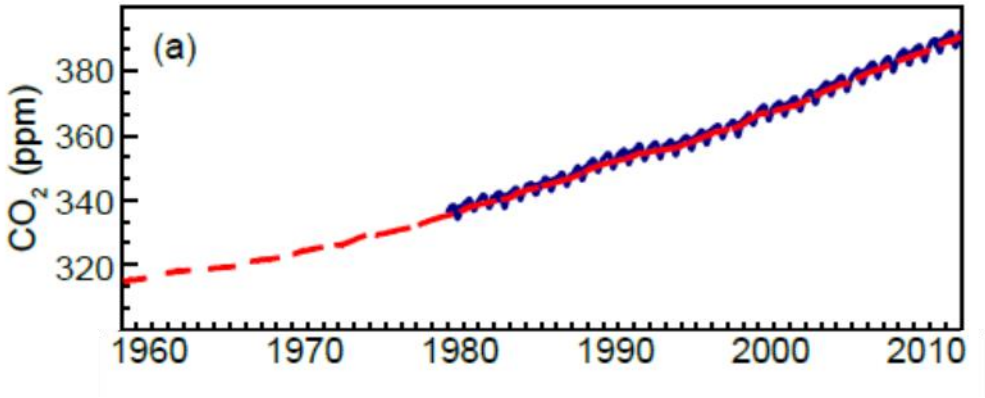

Fig.1.1: Increase in CO_2 levels in the atmosphere of Earth in the past decades (Taken from IPCC, 2013).

1.2 Carbon cycle

Carbon is one of the most important building blocks of life, meaning that it is constantly circulating where it can be released and re-absorbed (Figure 1.2). In the terrestrial biosphere when animals and plants die, they decay. The decomposition of their bodies is due to bacteria and fungi which convert most of the carbon into CO_2 or methane, making it part of the environment. Similar situations occur in the ocean, for example, when fish die. Over a very long period of time sedimentation occurs and it becomes part of the geosphere and this is how fossil fuels are produced. When carbon enters the ocean, bicarbonate is formed and organisms use it to make shells or limestone that sink to the bottom of the ocean where carbon can be stored (Detwiler & Hall, 1987; Sedjo, 2001; Benson *et al*., 2008; Ramanan *et al*., 2009; Graber, 2011).

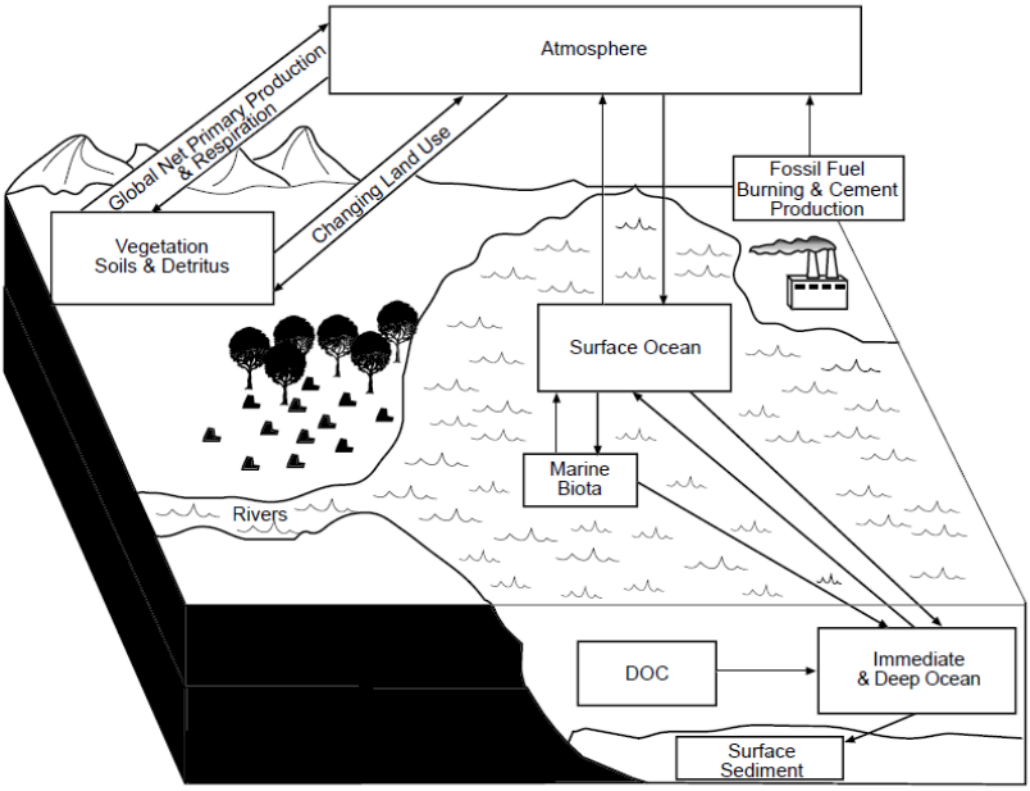

Fig.1.2. Global carbon flow between the terrestrial biosphere and the atmosphere (Taken from Schimel *et al.,* 1995).

1.2.1 Supercritical CO_2

Carbon dioxide usually behaves as a gas in air at standard temperature and pressure or as a solid state when frozen (in this form it is known as dry ice). When temperature and pressure are increased to above the critical point, the properties of CO_2 appear to be between a gas and a liquid. Supercritical carbon dioxide is a fluid state of CO_2 where critical temperature and pressure of 31˚C and 73 bar are attained or exceeded as can be seen in figure 1.3 (Morozova *et al*., 2010). Supercritical CO_2 has properties of a gas but the density of a liquid. Since CO_2 is non-polar, additional polar organic co-solvents can be added to the supercritical fluid for processing polar compounds. Therefore, a range of compounds, both polar and non-polar can be dissolved by

supercritical CO_2. Due to the low toxicity and environmental impacts and the role of chemical extraction, supercritical CO_2 is becoming an important commercial and industrial solvent. Most compounds can be extracted with minimal damage or denaturing, due to the stability of CO_2 and the low temperature of the process (Gupta, 2006). The solubility of CO_2 in CCS conditions will be approximately 0.33% (Carroll *et al*., 1991).

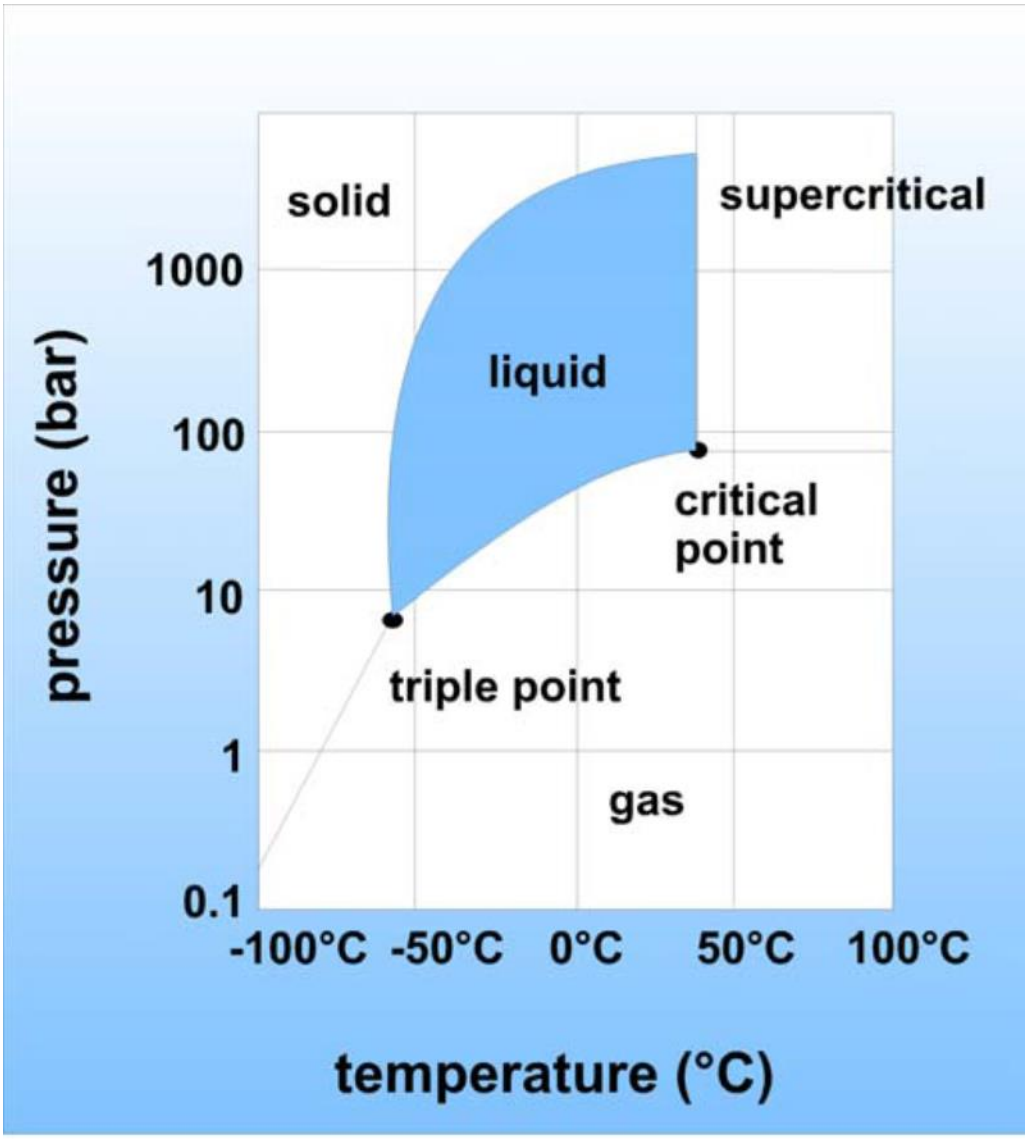

Fig.1.3. A phase diagram of CO_2 (Taken from ASCO CARBON DIOXIDE LTD).

1.3 Carbon sequestration

Carbon sequestration (CS) or carbon capture and storage (CCS) is a technology that can possibly prevent large quantities of CO_2 from being released into the atmosphere. The procedure involves capturing CO_2, using cleaning technology from large sources, followed by transporting and storing it deep underground so it does not have any contact with the atmosphere and minimizes climate change (Lal, 2008; The European CCS Demonstration Projects Network). The first step of CCS is to capture CO_2 released from large facilities, such as power plants. Once it is captured, the CO_2 is compressed to a liquid state and transported *via* pipelines, ships, or trucks to its final destination for long term storage. Two suggestions have been proposed for storing CO_2: firstly in the oceans and secondly in geological structures beneath the Earth's surface (Nye *et al*., 2007; Lotz & Brent, 2008; Peters, 2008). Of the two, geological sequestration, such as spent hydrocarbon reservoirs, depleted oil and gas and saline reservoirs and un-mineable coal beds, where it can store hundreds of billions of tons of CO_2, is likely to be more acceptable because it is easier to trace (Metz *et al*. 2005). The injected site is then measured, monitored and verified constantly to ensure that there is no leakage of CO_2. Figure 1.4 is a representation of the CCS system showing how CO_2 can be transported and stored (Ngô *et al*., 2004; Gilfillan *et al*., 2009; Ehlig-Economides & Economides, 2010; Viljeon *et al*., 2010).

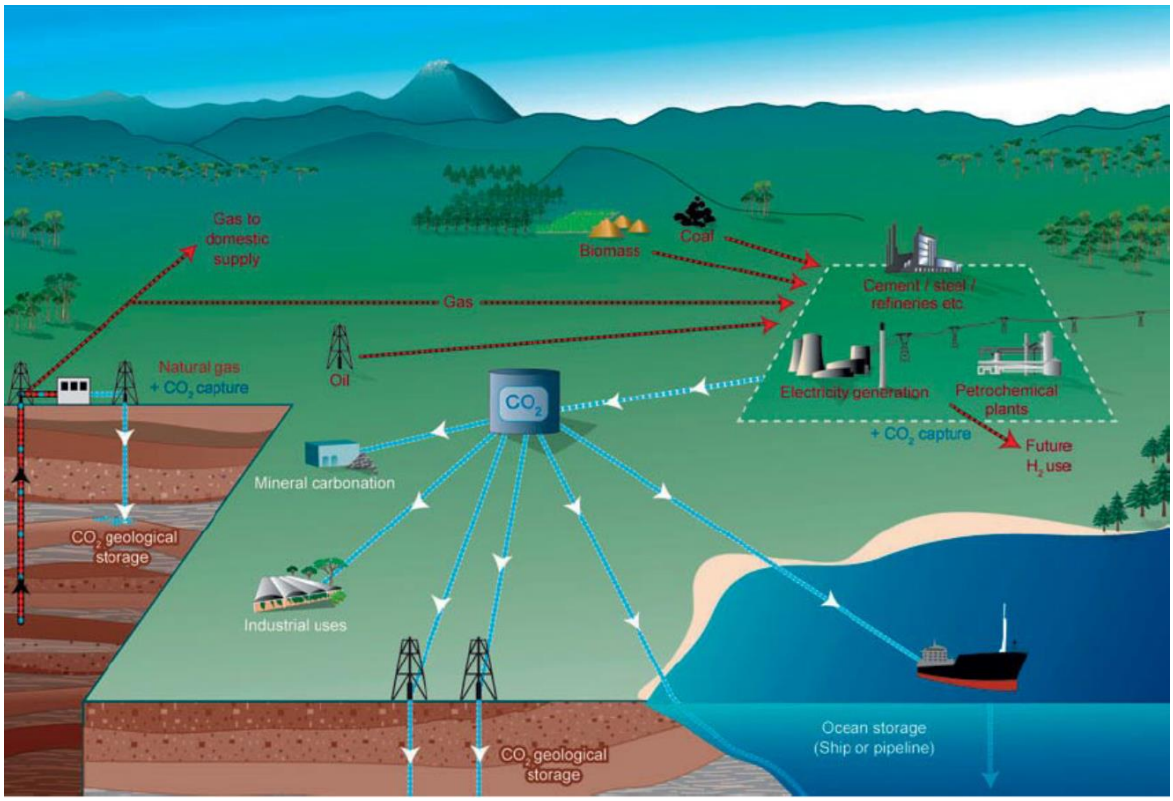

Fig.1.4. CCS system showing how CO_2 can be transported and stored (Taken from IPCC, 2005).

The best scenario is to store CO_2, and then to be able to reuse or make products such as paper filler, building materials, solar gasoline or enhanced oil recovery (EOR). In the case of EOR, CO_2 is injected to help the oil to flow more freely. Carbon capture and storage is assumed to be the most effective way of reducing CO_2 emissions (Lotz & Brent, 2008; CO_2 capture, transport and storage, 2009; Ehlig-Economides & Economides, 2010; West, *et. al*., 2011). Geological sequestration is currently being tested in some locations but more details of implementation such as materials issues, monitoring and controlling CO_2 migration, should be well understood to meet future challenges (Engelbrecht *et al*., 2004; Sheppard & Socolow, 2007). There have been several CO_2 sequestration demonstrations around the world. Table 1 represents a few of the storage projects.

Table 1: Storage projects during the past decade, showcasing variation in storage volumes and reservoir type (Taken from Peters, 2008).

Project name	Country	Injection start	Daily injection (tCO_2/day)	Total planned storage	Reservoir type
Weyburn	Canada	2000	3,000 - 5,000	20,000,000	EOR
In Salah	Algeria	2004	3,000 - 4,000	17,000,000	Gas field
Sleipner	Norway	1996	3,000	20,000,000	Saline formation
K12B	Netherlands	2004	100	8,000,000	EGR
Frio	United States	2004	177	1,600	Saline formation

1.3.1 Limitations of CCS technology

CCS is not a perfect technology, with one of the major disadvantages being the usage of additional energy to capture CO_2. Storage sites cannot be guaranteed for no leakage possibilities and finally the cost of CCS technology. No commercial scale projects have been integrated, therefore; costs are uncertain and limited information regarding introducing large amounts of CO_2 in geological areas is known (IPCC, 2005; Stavins & Richards, 2005).

1.3.2 Industrial CO_2 cleaning technologies

Three different types of CO_2 capturing technologies exist: pre-combustion, oxyfuel combustion and post-combustion. Pre-combustion involves heating fuel in a small amount of oxygen, which produces carbon monoxide and hydrogen known as ‘syngas’. Carbon monoxide is then converted to CO_2 with the addition of steam,

producing more hydrogen. CO_2 can then be chemically extracted; leaving hydrogen that can be used as a clean fuel in a power plant or other chemical processes. This technology has been applied in fertilizer, chemical and power productions. Pure oxygen is needed to burn fossil fuel for capturing CO_2 through oxyfuel combustion instead of air. Other gasses, such as nitrogen, are removed from the air to obtain oxygen. However, fuel gas consists mainly of pure CO_2 and water vapour, the latter of which is condensed through cooling. CO_2 can therefore be extracted and transported to storage sites (Engelbrecht *et al*., 2004; Global Climate & Energy Project An Assessment of Carbon Capture Technology and Research Opportunities, 2005; Lotz & Brent, 2008; Kanniche *et al*., 2010; The European CCS Demonstration Projects Network).

However, this technique has its shortcomings. For example, energy is required to operate the equipment needed to capture CO_2, resulting in the increase in electricity costs to capture CO_2 to 87% using the post-combustion capturing technique, 52% for the pre-combustion technique and an estimated increase in electricity cost of 32% for the oxyfuel combustion technique. However, to obtain pure oxygen for oxyfuel combustion capture, cryogenic cooling technology is required. As for post-combustion capture, energy is required to extract the CO_2 from the chemical solvent. It is known that the pre-combustion stream is potentially more efficient than post-combustion. Due to the energy required, most power plants are not fitted with CCS technology. It is more expensive to fit CCS in existing power plants than incorporating them into new plants. Designing power plants with CCS incorporated should reduce efficiency losses (Ngô *et al*., 2004; IPCC, 2005; Peters, 2008; CO_2 capture, transport and storage, 2009).

1.3.3 CO_2 transportation *via* pipelines

There have been major concerns about leakages that will compromise CCS as a climate change improvement option. However, transporting CO_2 *via* a pipeline has its disadvantages, such as, if a major fracture occurs in the pipeline due to failure or accidents, CO_2 would rapidly be released and cooled. This may result in formation of a vapour cloud around the fracture, eventually solidifying, affecting the characteristics of pure CO_2 and introducing additional complexities of the nature released CO_2. The cooling affect from the fracture can cause the area to become brittle, resulting in damage to the equipment. Transportation of CO_2 in a supercritical state is likely to be more desirable and would increase efficiency. This means that CO_2 will not turn to a liquid form no matter how much pressure is applied to the gas. In other words, CO_2 would be in a form known as a dense phase (high pressure liquid) (Ngô *et al*., 2004; Doctor *et al*., 2005; IPCC 2005; Brendan, 2007; CO_2 capture, transport and storage, 2009).

1.4 Storage options

1.4.1 Storage in oceans

Oceans, at present, are the largest carbon sink, absorbing more than a quarter of the carbon dioxide produced by humans. In future, oceans can be both a CO_2 source and sink. Several concepts have been proposed for ocean storage. Alternative storage options, including the use of chemical processes, can store CO_2 as a stable carbonate mineral form. This process is known as mineral carbonation or mineral

sequestration. Carbon dioxide can react with available metal oxides such as magnesium oxide (MgO) and calcium oxide (CaO) to form stable carbonates (O'Connor *et al*., 2000; Engelbrecht *et al*., 2004; Global Climate & Energy Project An Assessment of Carbon Capture Technology and Research Opportunities, 2005). Basalt storage offers a good form of oceanic carbon storage due to geothermal, sediment, gravitational and hydrate formation. CO_2 hydrate is denser than CO_2 in seawater. Injecting CO_2 at depths greater than 2,700 meters (8,900 ft.) will ensure that the CO_2 has a greater density than seawater, causing it to sink. Crushed limestone or volcanic rock can act as acid neutralisation, which naturally removes CO_2 from the atmosphere when added to oceans (Global Climate & Energy Project An Assessment of Carbon Capture Technology and Research Opportunities, 2005).

The disadvantage with oceanic and terrestrial storage is that high concentrations of CO_2 could negatively impact marine life by killing oceanic micro-organisms, which will then affect other forms of marine life. Dissolved carbon dioxide will most likely react with water, forming carbonic acid which increases the acidity of the oceans water; however, the environmental effect is poorly understood (Ocean Carbon Sequestration, 2007). CO_2 will also eventually equilibrate with the atmosphere so it is not a permanent storage option. Carbon can be stored on the seabed by growing oceanic phytoplankton blooms with iron fertilization. This approach can also be problematic because of the lack of understanding the effects on marine ecosystems such as the release of nitrogen oxides and the disruption of the ocean's nutrient balance (Copeland *et al*., 2003; Ngô *et al*., 2004; Lotz & Brent, 2008; Velea *et al*., 2009).

1.4.2 Storage in terrestrial environments

Grasslands contribute to soil organic matter, meaning soils can be an excellent carbon sink. The world's grasslands are mostly tilled and converted to croplands. Therefore, an increase of carbon sequestration in soil techniques such as no-till farming, cover cropping and crop rotation can be performed. Good management in grazing can sequester more carbon in the soil (Franzluebbers & Doraiswarmy, 2007). The Kyoto Protocol is an international agreement linked to the United Nations Framework Convention on Climate Change to reduce emission of CO_2, such as growing vegetation to absorb CO_2 (Lotz & Brent, 2008; Gorte, 2009). Agricultural sequestration has been alleged to have positive effects on soil quality, leading to increased food production (Rice & Fabrizzi, 2008). Forests can store up to 80% of carbon in soils as dead organic matter. There have been studies on tropical forests that showed 18% absorbance of CO_2 but also suggests that the forests temperate zones offers only a temporary cooling benefit (Wisniewskil *et al*., 1993; Sedjo, 2001; Engelbrecht *et al*., 2004; Ngô *et al*., 2004; Gilfillan *et al*., 2009). Geological sequestration also includes CO_2 storage underground, such as depleted oil, gas and saline reservoirs. These formations have the properties to dissolve CO_2 in groundwater that makes it possible for long term storage of CO_2. This storage option is environmentally effective and economically feasible with its own weaknesses and strengths. However, information regarding interactions between stored CO_2 and biomes underground is limited.

To secure the safety of storing CO_2 in geological formations, physical and chemical mechanisms are considered. These include depths between 600 m and 1 000 m,

which results in the CO_2 to exist as a supercritical fluid (Holloway, 2007). CO_2 in its supercritical state has a liquid-like density and a gas-like viscosity, and allows for more stable mineral compounds to be formed. The formation must then be monitored to minimize possible leaks because there are concerns about the effect of concentrated CO_2 on the local environment if leakage occurs underground, such as the oceans which can affect the marine ecosystem and microbial diversity terrestrially due to an increase in acidity (IPCC, 2005; CO_2 capture, transport and storage, 2009; Cunningham *et al*., 2009; West *et al*., 2011).

1.5 Carbon capture and storage in South Africa

CO_2 is an odourless gas, and is one of the most unwanted on the international climate change agenda. Developing countries such as South Africa, in common with other countries, are a coal-based energy economy. Energy demands are increasing around the world, resulting in an increase in CO_2 emission rates. Emissions are likely to continue, in spite of renewable energy programs and energy efficiency measures. South Africa is investigating the use of carbon capture and storage as a greenhouse gas emission improvement measure. Engelbrecht and co-workers (2004) showed that approximately 60% of CO_2 emitted per year is sequestratable. Therefore, with a CCS campaign in South Africa, it was important to make sure that there is potential in this technology. In 2004, the Department of Minerals and Energy commissioned an investigation pointed out that such potential does exist (Engelbrecht *et al*., 2004; Surridge & Cloete, 2009). It was decided that South Africa would concentrate on geological storage of CO_2. In 2009, the Department of Environmental Affairs announced that CO_2 emissions in South Africa will increase until 2020-2025 and

hopefully decrease after 2030-2035. A detailed Atlas on geological storage sites of CO_2 in South Africa was released in 2010. The CO_2 storage potential has been recognised in RSA, therefore, once the geology of a storage reservoir has been characterized (deep saline formation storage options onshore/offshore and deep coalfields of the Karoo Basin), a test injection in 2016 has been considered and a demonstration in 2020, to facilitate commercial operation by around 2025. CCS has been successfully used around the world and it may be a solution to climate change, but there is no doubt that this technology can reduce emissions of CO_2 (Cloete, 2010).

1.6 Life in the subsurface

The subsurface of our planet contains a great number of unknown life forms. However, the inner limits of our planet's life processes, the role of deep life in controlling biogeochemical processes and climate on the surface can be explored (Rampelotto, 2010; West *et al*., 2011). Little is known about the abundance, distribution, diversity and activity of the deep subsurface microbial life. Deep subsurface life has shown to continue living in complete isolation fixing its own carbon and nitrogen, and provides energy-yielding processes that sustain life, such as decomposition of water and producing H_2, O_2 and H_2O_2, with the subsequent oxidation of minerals containing reduced forms of sulphur, iron and manganese. Microbial activity in subsurface environments has the potential to play a critical role in cycling of carbon and other elements (Sogin *et al*., 2010).

As CCS is receiving more and more attention, so does the concern for long term storage with several questions that need to be answered. In the past few years there has been an increased recognition of the role of subsurface microbes. The production and modification of oil and gas deposits have raised questions about life in the deep subsurface (Engelbrecht *et al*., 2004). Excessive amounts of CO_2 have a large influence on life on the surface of the Earth. The question is what will happen if large amounts of CO_2 were stored in the subsurface and will this procedure disturb natural processes and create any risks in the subsurface? There are a few important questions that are needed to be answered. Little is known about the subsurface microbial communities and the critical carbon cycle processes. However, to answer these questions, the biochemistry and physiology of subsurface micro-organisms must be better understood and how carbons flow in the subsurface takes place. CO_2 cycling underground, microbial diversity, metabolic activities, interactions and CCS effects on biological turnover and cycling are poorly understood. Therefore, by understanding the deep subsurface biome with regard to CO_2 cycling, it can be determined what the possible consequences of long term storage of CO_2 underground can have on this biome (IPCC, 2005; Ménez *et al*., 2007). Related objectives for instance, measuring the chemosynthetic contributions to the global carbon cycle, determining how abiotic processes in the deep biosphere impact deep biology, the interactions between microbes and geological conditions, such as carbonates and weathering, and to determine the connections between deep life and global climate may also be an excellent research topic in the future (Sogin *et al*., 2010).

1.7 Conclusions

Global warming increases the average temperature of the Earth's atmosphere and causes climate change. As the world's population increases, so will the level of global warming due to demand in energy usage. CCS technology, which is a process that captures, transports and stores CO_2, can be a solution to minimize CO_2 emission into the atmosphere. Three different types of CO_2 capturing methods exist: pre-combustion, oxyfuel combustion and post-combustion. These technologies are currently being used in industrial applications. Two storage options are available which are oceans and terrestrial storages. However, transportation of CO_2 in a supercritical state is likely to be more desirable and would increase the efficiency of capturing the CO_2 in selected storage environments. In South Africa, terrestrial storage is of interest. Thus, there are a few questions needed to be answered. Questions such as what will happen to the microbial diversity underground once a large amount of CO_2 is injected and stored underground. Thus, by understanding the deep subsurface biome and its abilities to cycle, the survival of the subsurface biome under CCS conditions is also a crucial aspect that might influence other geochemical cycling in the subsurface. These aspects should also be considered as the consequences of long term storage of CO_2 underground could affect other natural geochemical processes.

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CHAPTER 2

CHAPTER 2

INTRODUCTION TO PRESENT STUDY

2. Introduction

High demands of coal usage in countries such as the United Kingdom, South Africa, China, the United States of America and India are causing rapid increases in concentrations of greenhouse gasses such as CO_2. This, in turn, is predicted to cause an increase in the average temperature of the Earth's atmosphere, contributing to climate change. In 2009, the Department of Environmental Affairs announced that CO_2 emissions in South Africa will increase until 2020-2025 and, hopefully, decrease after 2030-2035 (CO_2 capture, transport and storage, 2009; Cloete, 2010).

Carbon capture and storage (CCS) is a technology that captures, transports and stores emitted CO_2, which, in turn, can eliminate the contribution made towards climate change. There are two storage options available - ocean and terrestrial storage; however, terrestrial storage has not been studied extensively. Thus, the effects of storing CO_2 underground are still largely unknown. The Extreme Biochemistry group at the University of the Free State has been involved in characterizing the deep subsurface biomes for the past 15 years. In 2011 a new multidisciplinary grant (Alfred P. Sloan Foundation) that focuses on deep carbon cycling, characterizing the biogenic contribution in context of Deep Energy, expanded the knowledge of carbon metabolism in the subsurface. It is known that micro-

organisms, living in extreme environments, such as high temperature and anaerobic or acidic conditions, generally utilize different CO_2 fixation pathways (Johnston *et al*.,1999; Kharaka *et al*., 2009; Velea *et al*., 2009; Graber, 2011; West *et al*., 2011). This study aims to contribute to the understanding of how subsurface biomes will interact with sequestered carbon.

To address this question the sequestration conditions were simulated using a high pressure syringe incubator system. Micro-organisms that have been isolated from the deep subsurface, such as *Thermus scotoductus* SA-01, isolated by Kieft and co-workers (1999), *Geobacillus thermoleovorans* GE-7, isolated by DeFlaun and co-workers (2007) and *Geobacillus thermoparaffinivorans* A12, isolated by Jugdave (2011), were selected for this study. As the control, a microorganism that is known to grow at 2 bar pressure and utilize CO_2, *Eubacterium limosum* (Genthner *et al*., 1981) was selected. These micro-organisms were used to simulate exposure to terrestrial carbon sequestration conditions.

2.1 Main objectives

The objectives of this study were to assess the effects that CCS conditions and CO_2 exposure might have on the selected micro-organisms. This study used molecular techniques to identify micro-organisms and basic genome mining was used to compare their metabolic capabilities, focussing on carbon fixation.

High pressure systems, that simulate the terrestrial sequestration parameters, were used to study survival and possible metabolic capabilities. Pressures from 2 to 100

bar were introduced and characterizations, using selective analytical techniques, for example live/dead staining, metabolic tests, High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) were carried out to determine if these microorganism can withstand increasing pressure and fix carbon dioxide.

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CHAPTER 3

CHAPTER 3

MOLECULAR IDENTIFICATION AND GROWTH STUDIES OF SELECTED MICRO-ORGANISMS

3. Introduction

Carbon sequestration (CS) or carbon capture and storage (CCS), as described in chapter 1, is associated with the deep subsurface (Herzog *et al*.,1992; Metz *et al*., 2005; Santillan *et al*., 2013). In geological formations, especially at depths greater than 600 m to 1000 m, the association of CO_2 with pressure will determine its characteristics that facilitate storage (Holloway, 2007).

The subsurface is known to possess one of the largest habitats for a high number of different groups of micro-organisms. CO_2 storage sites beneath the Earth's surface could directly affect the deep subsurface microbial ecosystems and biogeochemical processes. Microbes found underground can survive in extreme environments with limited nutrient and energy supplies, resulting in very low metabolic rates (Lin *et al*., 2007; Roussel *et al*., 2008; West *et al*., 2011). The understanding of carbon capture and storage in the deep biosphere' and the behaviour of CO_2 are limited. Consequently, it is important to evaluate the potential effect of CO_2 on the microbial population in the deep subsurface (Herzog *et al*., 1992; West *et al*., 2011). Therefore, understanding more about the carbon cycle associated with terrestrial subsurface biomes, will contribute to our understanding if interactions between available CO_2 and the microbial population will occur.

3.1 Deep subsurface microbes

The deep subsurface microbial diversity is responsible for a large portion of the biomass on the planet (Pfiffner *et al*., 2006). They carry out processes that can alter the chemical makeup of minerals as well as the mineral content of groundwater, and can degrade pollutants (Lin *et al*., 2007). The life cycles of these microbes are impressively slow and some microbes remain metabolically dormant for an extended period. The largest limitation for the deep subsurface microbes is the increase of temperature with depth and the concomitant decrease of nutrients, both of which cause the metabolic rate of the microbial communities to significantly slow down (Lovley & Chapelle, 1995; Reith, 2011).

Microbial communities in the deep subsurface are very diverse (Krumholz, 2000). The communities consist mainly of bacterial and archaeal species that focus on inorganic substrate oxidation, with iron and sulphur oxidation as the two main energy sources. Due to lack of oxygen, the deep subsurface microbes have engaged in anaerobic respiration where NO_3^-, SO_4^{2-} and CO_2 can serve as the terminal electron acceptor. The deep subsurface microbes are known to reduce inorganic compounds found in the rock. Microbes are also able to utilize H_2 gas, SO_3^{2-}, $S_4O_6^{2-}$, S^0, Fe^{2+}, and Mn(II) as an electron donor/acceptor. They are also capable of arsenic oxidation and in some cases reduction of organic compounds in oil or sediments. They can also utilize hydrocarbons for energy and use CO_2 trapped in the rocks as their carbon source. Thermophilic micro-organisms that oxidize metals, methanogens, anaerobic heterotrophs, autotrophic lithotrophs and radiation-resistant microbes thrive in deep subsurface environments. These organisms constitute the largest portion of biomass

in the deep subsurface biosphere (Krumholz, 2000; Reith, 2011).

3.2 Genus *Thermus*

The genus *Thermus*, which means hot, are not restricted to natural environments and have been isolated from numerous areas such as artificial thermal environments to abyssal geothermal sites (Kristjansson *et al*., 1994; Chung *et al*., 2000; Guo *et al*., 2003). The *Thermus* ecology are associated with photosynthetic and chemolithotrophic prokaryotes which makes it a good candidate for carbon capture and storage environments. This genus includes a high diversity of thermophilic and extreme thermophilic strains distributed around the world (Cava *et al*., 2009) and it is one of the most wide spread genera of thermophilic bacteria, with isolates found in natural as well as in man-made thermal environments (Kristjansson *et al*., 1994). *Thermus* species are generally found in neutral to slightly alkaline, natural aquatic environments with temperatures ranging between 50 and 85˚C, are amenable to genetic manipulation and is closely related to the mesophilic, radiation-resistant *Deinococcus radiodurans* (Jenney & Adams, 2008). However, studies have proven that *Thermus* isolates can grow anaerobically, using nitrate as the terminal electron acceptor (Williams & Sharp 1995; da Costa *et al*. 2001).

Kieft and co-workers (1999) isolated *Thermus scotoductus* SA-01 in 1999 from a South African gold mine at a depth of 3.2 km. This strain is closely related to *Thermus* sp. strains NMX2, A1 and VI-7, isolated from thermal springs in New Mexico, USA, and Portugal (Balkwill *et al*., 2004). *T. scotoductus* SA-01 has been characterized as a facultative anaerobe capable of coupling the oxidation of organic

substrates to reduce a wide range of electron acceptors, including O_2, nitrate, Fe(III), Mn(IV), S^o, Co(III)- EDTA, Cr(VI) and U(VI). Nearly all *T. scotoductus* strains are capable of dissimilatory Fe(III) reduction (Kieft *et al*., 1999).

T. scotoductus SA-01 is a facultative heterotrophic, thermophilic anaerobe. It is a Gram-negative bacterium that is non-motile and non-sporulating, rod or slender like cells. They most likely form septated filaments in the exponential phase under optimal conditions and subsequently separate by binary fission when it reaches the stationary phase (Cava *et al*., 2009; Gounder *et al*., 2011). This bacterium grows in extreme environments under both aerobic and anaerobic conditions, with optimum growth temperature of 65˚C at pH 7 and no requirement of specific amino acids or vitamins (Kieft *et al*., 1999). This organism is oxidase- and catalase positive, and is able to utilize a wide spectrum of carbohydrates in the presence of either nitrate or oxygen (Jenney & Adams, 2008; Cava *et al*., 2009; Pati *et al*., 2011).

3.3 Genus *Geobacillus*

Moderate thermophilic or hyperthermophilic micro-organisms, belonging to the genus *Bacillus*, were reclassified as a new genus, named *Geobacillus*, a decade ago (Zeigler, 2001). Due to *Geobacillus* isolates containing similar fatty acid compositions, several biochemical characteristics and cell morphology such as Gram-positive cell wall structure, sugars and high GC content, slight variations in the saturated and unsaturated fatty acids and DNA composition, can assist in the new taxonomic position of a novel species or strain belonging to this genus (Nazina *et al*., 2004, 2005).

Thermophilic *Bacillus* species, such as *Geobacillus* that have ubiquitous capabilities to metabolize hydrocarbons, and have been successfully isolated from geothermal areas from all continents. Isolation sites included shallow marine hot springs, deep-sea hydrothermal vents, high temperature oil fields, artificial hot environments such as hot water pipelines and heat exchangers as well as water treatment plants, coal burning refuse piles and bioremediation biopiles (Sharp *et al*., 1992; Maugeri *et al*., 2001).

Geobacillus sp. A12, isolated from the Northern platinum mine (now renamed Zondereinde division), situated in the upper end of the western limb of the Bushveld Igneous Complex (BIC), shows 99% identity to *Geobacillus stearothermophiles* (Jugdave, 2011). The optimal growth parameters for this isolate is 55˚C, pH 7 and a variety of carbon substrates such as L-Arabinose, D-Ribose, D-Trehalose, D-Xylose, α-ketovaleric acid, L-Malic acid, pyruvic acid, methyl ester and succinic acid mono-methyl ester (Jugdave, 2011).

Geobacillus thermoleovorans GE-7 was isolated from the West-Driefontien gold mine in South Africa at a depth of 3.2 km, from dripping water fracture at pH 8.0, 60˚C. It is a Gram-positive, facultative aerobic microorganism that is rod-shaped with terminal endospores and flagella. The optimal growth conditions for this isolate are at 65˚C, pH 7.5. This species is also able to grow on a variety of carbon substrates such as triacylglycerides (ranging from C4 to C18), including cellobiose, hydrocarbons and lactate. This isolate can utilizes nitrate and O_2 as an electron acceptor, which means this organism is adapted to the oxic/anoxic interface and is also radiation resistant (DeFlaun *et al*., 2007).

3.4 Genus *Eubacterium*

Different strains of *Eubacterium limosum* have been isolated from a variety of habitats, including the human intestine, sewage, rumen of sheep, soil and anaerobic digesters (Leclerc *et al*., 1997). *E. limosum* is a non-sporulating, acetogenic bacterium that grows chemolithotrophically under anaerobic conditions, with pressure up to two bar at 34-37˚C and pH 7.0-7.2. This microorganism can reduce CO_2 as the terminal electron acceptor, forming acetate and butyrate through an energy conserving process on various substrates, making it a good candidate for CCS environments (Genthner *et al*., 1981; Mŭller *et al*., 1981). Previous studies have shown that *E. limosum* utilizes carbon-one compounds, such as methanol, CO and H_2-CO_2 as energy sources, to produce acetate and long chain fatty acids. In the presence of CO_2, this organism is able to convert methanol to acetate (Chang *et al*., 1999; Chang *et al*., 2001).

3.5 Aims of this chapter

The main aims of this chapter were to:

- Confirm the identity of the selected micro-organisms that could be exposed to CCS conditions.
- Grow the selected micro-organisms under various parameters such as aerobic, anaerobic and anoxic conditions.
- Monitor their survival and metabolic activities.

3.6 Materials and methods

3.6.1 Medium preparation and growth conditions

Bacterial strains used in this study are listed in table 3.1. Isolates were kindly supplied by Prof. Mary DeFlaun (Geosyntec Consultants Inc.) (GE-7), Abhita Jugdave (Ph.D. study, Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, R.S.A) (A12), Prof. Tom Kieft (University of Free State/ New Mexico Tech) (SA-01) and *E. limosum* (DSM-20543) was bought from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). These micro-organisms were selected for growth in aerobically, anaerobically and anoxic conditions. Glycerol stocks of the cultures were taken from -80˚C and revived in their respective media for growth studies and identification.

Table 3.1: Bacterial isolates and their known characteristics.

Organism	Area	Temperature	pH	Characteristics	Medium[A]
***Thermus scotoductus* SA-01** (Kieft *et al*., 1999)	Deep gold mine (South Africa)	65˚C	7	Facultative anaerobe	**TYG medium** (5 g/L tryptone, 3 g/L yeast extract, 1 g/L glucose), pH 7
***Geobacillus thermoleovorans* GE-7** (DeFlaun *et al*., 2007)	Driefontein mine (South Africa)	65˚C	7.5	Facultative anaerobe	**R2A medium** (0.5 g/L yeast extract, 0.5 g/L peptone, 0.5 g/L glucose, 0.5 g/L casamino acids, 0.5 g/L starch, 0.3 g/L sodium pyruvate, 0.3 g/L potassium phosphate, 0.05 g/L magnesium phosphate), pH 7.5
***Geobacillus thermoparaffinivorans* A12** (Jugdave, 2011)	Northern platinum mine (South Africa) (Zondereinde)	55˚C	7	Facultative anaerobe	**LB medium** (10 g/L peptone, 5 g/L yeast extract, 10 g/L sodium chloride), pH 7
Eubacterium limosum (Genthner *et al*., 1981; Chang *et al*., 1999)	Variety of habitats (DSMZ)	34˚C	7.2	Strict anaerobe Can grow at 2 bar	**PYG medium** (5 g/L trypticase peptone, 5 g/L peptone, 10 g/L yeast extract, 5 g/L beef extract, 5 g/L glucose, 2 g/L K_2HPO_4, 1 ml tween 80, 0.50 g/L [B]cysteine-HCl, 1 mg/L [B]resazurin, 40 mL salt solution, 950 mL distilled water, 10 mL haemin solution, 0.20 mL vitamin K_1 solution), pH 7.2

[A] Plates were prepared by adding additional 1.6% (w/v) agar to the respective media and then autoclaved. Anoxic mediums were prepared by adding additional 10 mM KNO_3^- as a terminal electron acceptor and 0.0003% (v/v) of resazurin from a stock solution of a 0.1% (w/v) as an oxidation-reduction indicator to the respective media and then autoclaved. Anaerobic media were prepared the same as anoxic media, but with addition of gas tight serum vials, sealed with rubbers, clamped with metal caps (Wheaton science products, U.S.A) and degassed for 1 hour while using 30 alternating cycles to introduce a nitrogen headspace. Then finally 10 µL of a 2.5% (w/v) stock solution of cysteine-HCl to remove the remaining oxygen (Sambrook *et al*., 1989; DeFlaun *et al*., 2007).

[B] Care was been taken for the preparation of the 0.1% (w/v) resazurin and the 2.5% (w/v) cysteine-HCl stock solutions. The pH of the 2.5% (w/v) cysteine-HCl was adjusted to 1.5-2, filter sterilized, added into autoclaved serum vials and degassed as described before for 1 hour while using 10 alternating cycles. The cysteine-HCl and resazurin was then stored at 4˚C.

3.6.2 Growth studies

Bacterial cultures (Table 3.1) were revived by inoculating 1 mL from a -80˚C glycerol stock into test tubes containing 4 mL of their respective media and allowed to grow overnight. The 5 mL overnight culture was then inoculated into 45 mL media and the OD_{600} readings (Spectronic® GENESYS 5) were recorded every hour for the aerobic growth studies and every four hours for the anaerobic and anoxic growth studies. All experiments were performed in duplicate.

3.6.3 Gram staining

Cell morphology verification analyses was done using the Gram stain procedure (Bartholomew & Mittwer, 1952) and evaluated microscopically at 1000 X magnification. Gram staining was performed by fixing a small amount of the culture from section 3.6.2 onto a microscope slide, followed by flooding the entire slide with crystal violet for one minute. The slide was rinsed with water and then an iodine solution was used to flood the slide for one minute before the slide was again rinsed with water. Ethanol [95% (v/v)] was then added drop-wise until the blue colour was no longer visible from the sample. The sample was then rinsed with water. The final step was to use Safranin red, which was a counter stain, to flood the slide for one minute and again rinsed with water to remove any excess dye. The slide was air dried and microscopically analysed (Bergey *et al*., 1994).

3.6.4 Live/dead staining

Live/dead staining was performed according to manufacturer's instructions (LIVE/DEAD® BacLight. Bacterial Viability Kits) Cat# L7007 (Molecular Probes, Inc.). A Live/dead stain was performed to determine if the cells remained viable during growth studies where live cells stained green and dead cells stained red because of the propidium iodide penetrating damaged bacterial membranes.

3.6.5 Nitrite formation

The nitrite stock solution of 1 mM (provided by the manufacturer) was diluted with distilled water to construct a nitrite standard curve (Figure 3.1), by plotting the absorbance at 548 nm against the known nitrite concentrations in µM. This standard curve was constructed to calculate the concentration of nitrate being reduced using the Griess Reagent Kit (G-792; Griess, 1879) (Invitrogen).

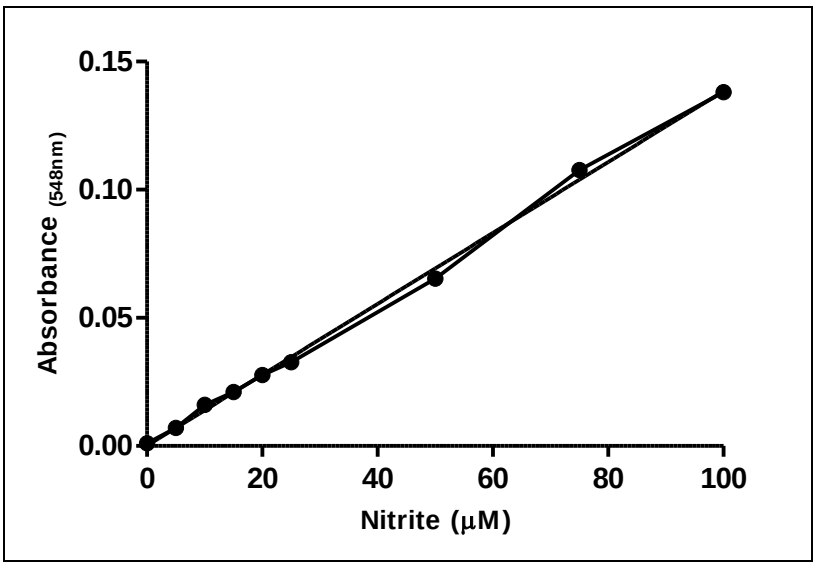

Fig.3.1. Nitrite standard curve, indicating the relationship between the nitrite concentration and absorbance at 548 nm (R^2= 0.9959). Standard deviations are smaller than the symbols used.

3.7 Genomic DNA extraction

Cells were harvested (1 mL) from the 50 mL culture (section 3.6.2) by centrifugation at 10 000 *x g* (Eppendorf, Centrifuge 5424) for ten minutes. Genomic DNA was extracted using the NucleoSpin® Soil Genomic extraction kit (Macherey-Nagel), according to the manufacturer's instructions, and stored at −20˚C. The concentration was determined by using the NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE). The genomic DNA that was isolated from each of the four micro-organisms was used as template for PCR amplification of an approximately 1500 base pair (bp) segment of the 16S rRNA gene as described in section 3.8.1.

3.7.1 Gel Electrophoresis

Genomic DNA was loaded on a 0.8% (w/v) or 1% (w/v) agarose gel (depending on the application), containing 2.5 mg/µL ethidium bromide (EtBr). The gel was prepared by weighing 0.8 or 1 g of agarose powder and poured into a 500 mL flask; 100 mL of TAE Buffer (40 mM Tris-HCL, 20 mM acetic acid, 1 mM EDTA, pH 8) was added to the flask. The GeneRuler™ DNA ladder (Fermentas) and the samples, containing loading buffer, were pipetted into separate wells in the gel. After electrophoresis at 90 volts for 45 minutes, the gel was removed from the tray and visualized with a ChemiDoc XRS (Bio-Rad Laboratories) gel documentation system. The GeneRuler™ DNA ladder (Fermentas) was used to infer the size of the DNA containing sample bands.

3.8 Confirmation of bacterial strains identity

3.8.1 PCR amplification using gradient PCR

For bacterial 16S rRNA gene amplification, each polymerase chain reaction (PCR) reaction contained 1 µL genomic DNA (50-100 ng), 0.25 µL of NEB *Taq* DNA polymerase (5U/ µl), 1 µL of dNTPs (10 mM), 1 µL forward primer 27F (10 µM) and 1 µL reverse primer 1492R (10 µM) shown in table 3.2, 1 µL of a 10 mg/mL BSA or DMSO solution and 5 µL of 10X (New England BioLabs) buffer. Each reaction was made up to 50 µL using sterile milliQ water. Each PCR reaction consisted of an initial denaturation at 94˚C for two minutes, followed by 30 cycles of 30 seconds denaturation at 94˚C, 45 seconds annealing at a gradient of 41-52˚C, and 90 seconds extension at 72˚C, and a final extension at 72˚C for seven minutes (Lane 1991).

Table 3.2: Universal primer sequences for bacterial 16S rRNA gene amplification.

Primers	Sequence	Reference
27F 1492R	5'- AGA GTT TGA TCM TGG CTC AG-3' 5'- GGT TAC CTT GTT ACG ACT T-3'	Lane, 1991

Annealing temperatures of between 41-52˚C were evaluated. The optimal annealing temperature was then used to run PCR amplification. After PCR amplification, the 16S rRNA gene products were resolved on a 1% (w/v) agarose gel, as described in section 3.7.1. For DNA cloning purposes, the gel was visualized with a DarkReader™ transilluminator, facilitating the excision of the DNA from the gel. The fragments on the gel were recovered with a blade and placed into 1.5 mL eppendorf tubes for gel extraction. The BioSpin Gel Extraction Kit was used to purify DNA fragments from the

agarose gel in TAE buffer as specified by the manufacturer (Biospin Gel extraction Kit Cat# BSC02S1 by Bioflux). The concentration of the purified PCR products were determined as described in section 3.7.

3.8.2 Cloning 16S rRNA gene into pGEM®-T Easy vector

The purified PCR products (section 3.8.1) were ligated into the pGEM®-T Easy vector system (Figure 3.2) according to the manufacturer's instructions (Promega Corporation, USA). All ligation reactions were performed by adding 3.9 µL of the purified PCR product (10-50 ng), 0.6 µL of pGEM®-T Easy vector (50 ng), 1 µL of T4 DNA ligase (1 U, Fermentas), 1 µL of 1x T4 ligase buffer and 3.5 µL of sterile milliQ water to make up a total of 10 µL. This was followed by incubating the ligation reaction at 4˚C overnight (Bester *et al*., 2010). The product (5 µL) was then transformed into *Escherichia coli* Top 10 competent cells (Invitrogen, U.S.A).

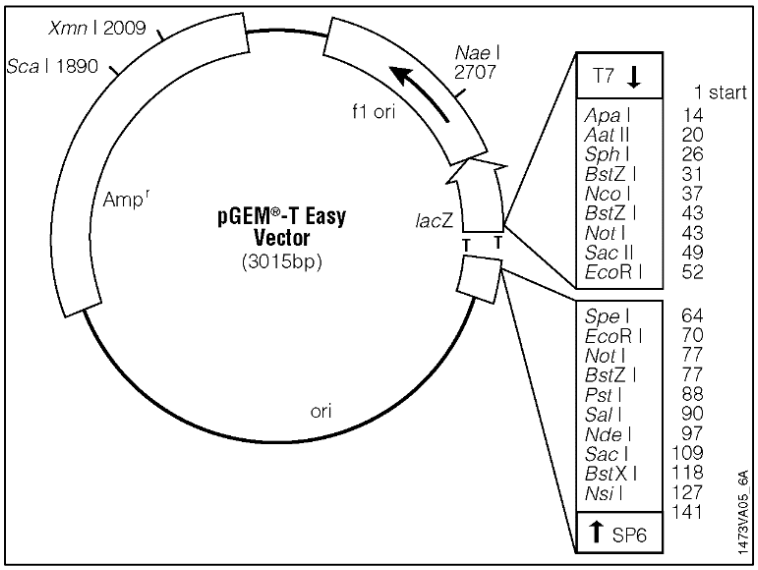

Fig.3.2. pGEM®-T Easy Vector System (Promega).

3.8.3 Competent cells

Competent *E. coli* Top 10 cells were prepared in aliquots of 50 µL using the method described by (Hanahan, 1983). In short, a pre-inoculum was grown overnight at 37˚C (5 µL glycerol stock in 5 mL LB medium). The overnight culture (1 mL) was inoculated into 100 mL Psi-broth (5 g/L yeast extract; 20 g/L tryptone; 5 g/L $MgSO_4.7H_2O$, pH 7.6) and grown to an OD_{600} of 0.8-1. The cells were placed on ice for 15 minutes and all the steps afterwards were performed on ice. Cells were centrifuged (Allegra™ 25R centrifuge, Beckman Coulter™) at 4000 *x g* for five minutes. The pellet was re- gently suspended in 40 mL ice cold TFB1-buffer (50 mM manganese chloride; 30 mM potassium acetate; 100 mM rubidium chloride; 10 mM calcium chloride; 15% (w/v) glycerol, pH 5.8). The cells were then incubated on ice for 15 minutes and centrifuged at 4000 *x g* for five minutes at 4˚C, where after the pellet was re-suspended extremely gently in 4 mL TFB2-buffer (10 mM MOPS; 75 mM calcium chloride; 10 mM rubidium chloride; 15% (w/v) glycerol, pH 6.5). The cells were then incubated on ice for 60 minutes, dispensed (50 µL) in 1.5 ml tubes, snap frozen with liquid nitrogen and stored at -80˚C (Hanahan, 1983).

3.8.4 Transformation

Competent *E. coli* Top 10 (Invitrogen, Carlsbad, CA) cells were thawed on ice. The ligation product (5 µL) from section 3.8.2 was then added to the competent cells and incubated on ice for one hour. After one hour the cells were heat-shocked at 42˚C for 35 seconds where after it was incubated on ice for two minutes before adding the 250 µL of LB medium supplemented with 50 µL 0.02 M Mg^{2+} and 100 µL 1 M glucose

solution. The cells were incubated at 37˚C for one hour on a shaker rotating at 175 rpm. The transformation mix (355 µL) was then plated onto AIX plates (LB medium, 10g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) supplemented with 10 mg/mL ampicillin, 24 mg/mL IPTG [isopropylthio-β-D-galactoside], 20 mg/mL X-gal [5-bromo-4-chloro-3-indolyl-β-D-galactoside] and 15 g/L agar) and incubated at 37˚C overnight. Single white colonies were selected and inoculated into 5 mL LB media, supplemented with 10 mg/mL ampicillin and incubated at 37˚C overnight. Plasmid DNA was isolated using the Biospin Plasmid DNA Extraction Kit (Biospin Plasmid DNA extraction Kit Cat# BSC01S1 by Bioflux) according to the manufacturer's specifications. Concentrations of the plasmids were determined as described in section 3.7.

3.8.5 Evaluation of the 16S rRNA gene inserts

The 16S rRNA gene sequences of *Geobacillus thermoparaffinivorans* A-12 accession number EU214615.1, *Geobacillus thermoleovorans* GE-7 accession number AY450926.1, *Thermus scotoductus* SA-01 accession number EU330195.1 and *Eubacterium limosum* ZL2 accession number JQ085755.1, were retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov/). *In silico* analysis was performed using restriction mapper (http://www.restrictionmapper.org) to confirm that the restriction enzymes *Nco*I and *Sal*I do not digest any of the four 16S rRNA gene sequences. Purified plasmid DNA was subjected to restriction enzyme digestions to confirm the presence of the ligated insert. The restriction digests of 20 µL were then incubated at 37˚C overnight and the products were evaluated on a 1% (w/v) agarose gel. Positive ligation of the 16S rRNA gene insert into the plasmid backbone was confirmed with the presence of the ~3000 bp plasmid backbone and the ~1500 bp insert (Figure 3.17).

3.8.6 Selection of positive clones for sequencing

A number of positive clones, obtained in the previous section, were selected for DNA sequencing to verify the identity of the bacterial strains, using the SP6 and T7 primers (Table 3.3). Sequencing was performed with the BigDye terminator v. 3.1 Cycle sequencing kit, according to the manufacturer's instructions. The sequencing reactions were as follows: 0.5 µL Premix, 1 µL of 3.2 *p*mol.µ/L sequencing primer T7 and 1 µL of 1.6 *p*mol.µ/L sequencing primer SP6, 2 µL dilution buffer, ~200 ng DNA template and sterile milliQ water to make up a total volume of 10 µL. The sequencing reaction was as follows: denaturing temperature was 96˚C for ten seconds, primer annealing at 50˚C for five seconds and amplification temperature was 60˚C for four minutes for 25 cycles (Kieft *et al*., 1999).

Table 3.3: Primer sequences for the pGEM®-T Easy vector and insert sequencing.

Primers	**Sequence**
T7 Promoter	5'-TAATACGACTCACTATAGGG-3'
SP6 Promoter	5'-TACGATTTAGGTGACACTATAG-3'

3.8.7 Sequence PCR purification

The sequencing products from section 3.8.6 were purified using the EDTA/ethanol precipitation post-reaction clean-up. The sequencing reaction volume was adjusted to 20 µL with sterile milliQ water and transferred to a 1.5 mL Eppendorf tube that contained 5 µL 125 mM EDTA and 60 µL of 100% (v/v) absolute ethanol. The tube was vortexed for five seconds and the solution precipitated at room temperature for 15 minutes, followed by centrifuging at 4˚C (Allegra™ 25R centrifuge, Beckman Coulter ™)

for 30 minutes at 20 000 *x g*. The supernatant was completely aspirated without disturbing the pellet. Then 200 µL of 70% (v/v) ethanol was added to the tubes and centrifuged at 4˚C for 30 minutes at 20 000 *x g*, (this step was performed twice). The supernatant was completely aspirated followed by drying under vacuum (Eppendorf concentrator 530) for five minutes. The samples were stored at 4˚C. The products were then sequenced with BigDye v.3.1 Cycle at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State (RSA). The 16S rRNA gene sequences were aligned against the non-redundant nucleotide GenBank database by using the BLAST (Basic Local Alignment Search Tool), BLASTn algorithm at NCBI (Altschul *et al.,* 1990) (National Centre for Biotechnology Information) shown in supplement A (Section 3.12).

3.9 Results and discussion

3.9.1 Aerobic growth

Experiments were set up as described in sections 3.6.1 and 3.6.2. According to literature, *E. limosum* is a strict anaerobe that can grow at 2 bar pressure (Genthner *et al*., 1981). Therefore, no aerobic growth was shown with this microorganism. The graphs in figure 3.3 A-C represent aerobic growth studies on the three selected mine micro-organisms. It takes *T. scotoductus* SA-01 approximately 13 hours to reach late exponential phase (Blue line, figure 3.3 A), *Geobacillus* sp. A12 approximately 6 hours (Red line, figure 3.3 C) and *Geobacillus* sp. GE-7 approximately 9 hours (Green line, figure 3.3 B). Specific growth rate (μ_{max}) is the number of cells by which the population increases per unit time. To calculate μ_{max}, equation 3.1 was used (Krishnaiah *et al*.,

2006; Widdel, 2010).

$[\ln (x_t/x_0) = \mu.t]$ (Equation 3.1)

(x_0) = initial concentration

(μ_{max}) = how fast and long the cell is growing

(t) = time it took to grow

(x_t) = cell concentration at the time given

Specific growth rate for *T. scotoductus* SA-01 was 0.15 per hour, *Geobacillus* GE-7 (Tlou, 2010) was 0.37 per hour and *Geobacillus* A12 was 0.97 per hour. All studies were performed in duplicate.

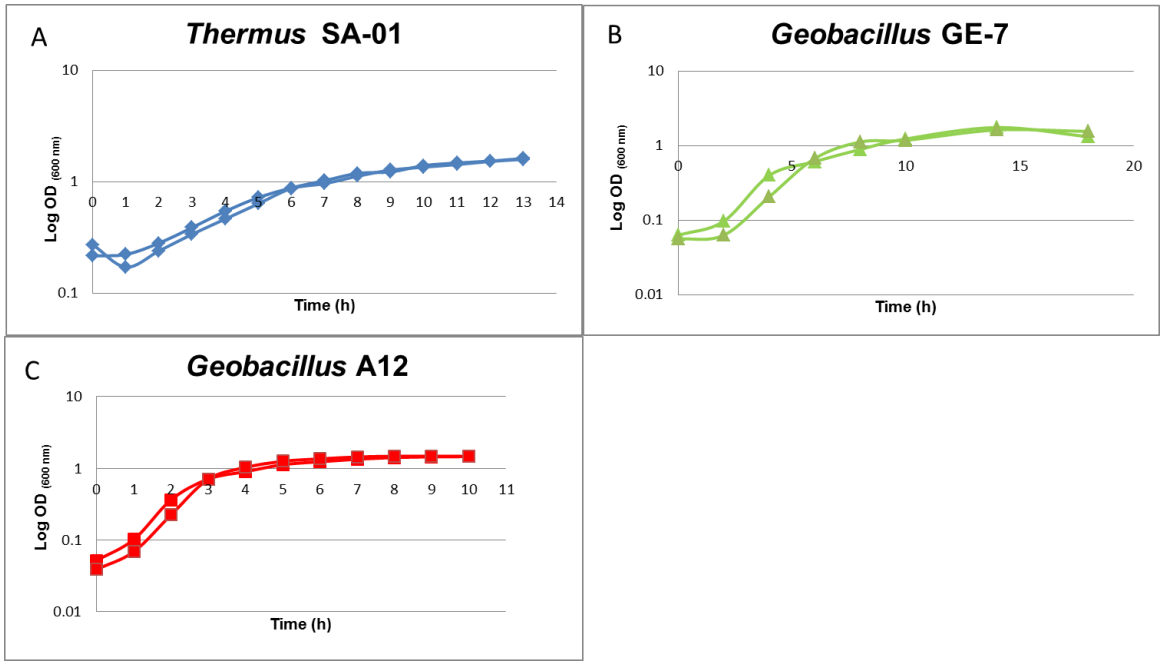

Fig.3.3. Aerobic growth curves for the three selected micro-organisms where optical density was monitored over time. *T. scotoductus* SA-01 (A) (Blue line), *Geobacillus* sp. GE-7 (B) (Green line) and *Geobacillus* sp. A12 (C) (Red line).

3.9.2 Gram staining

Microscopic cell differentiation analyses were performed as described in section 3.6.3. The selected isolates were both Gram-positive and Gram-negative bacteria with rod shape morphology, presented in figure 3.4 A-C and figure 3.5. A Gram-negative micro-organism does not have a peptidoglycan layer which therefore does not retain the crystal violet dye when compared to the Gram-positive micro-organisms that stains purple. Gram-negative micro-organisms are equipped with a lipopolysaccharide layer and a thin cell wall (Bergey *et al*., 1994). Results showed that *T. scotoductus* SA-01 is a Gram-negative rod (Figure 3.4 A), *Geobacillus* sp. A12 is a Gram-positive rod (Figure 3.4 B), *and Geobacillus* sp. GE-7 is a Gram-positive rod (Figure 3.4 C).

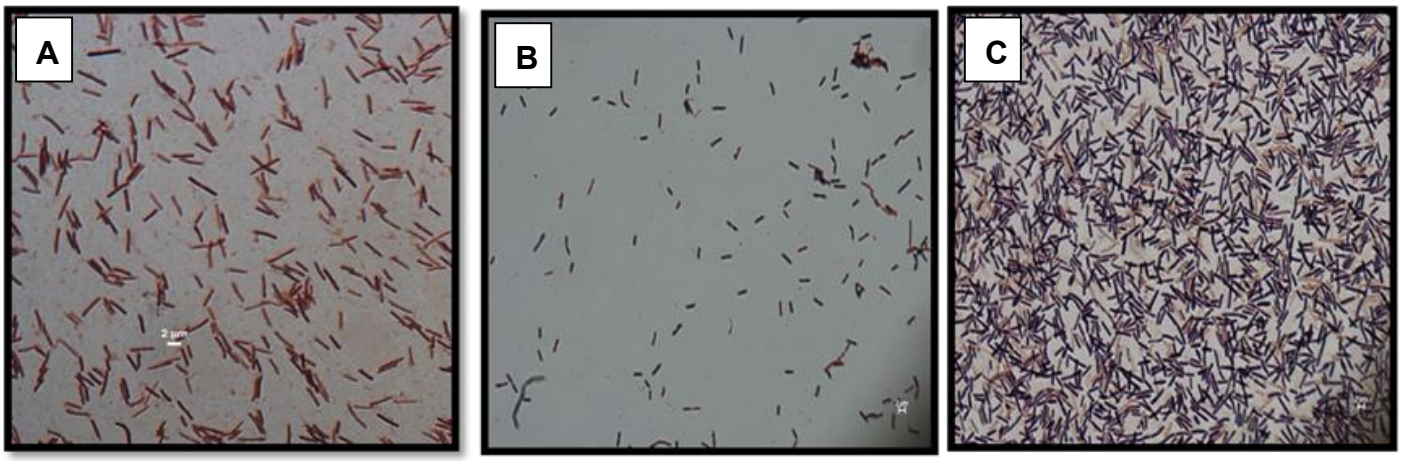

Fig.3.4. Gram staining characteristics of the three mine isolates. Scale bars were set at 2 µm. *T. scotoductus* SA-01 is a Gram-negative rod (A), *Geobacillus* sp. A12 is a Gram-positive rod (B), *Geobacillus* sp. GE-7 is a Gram-positive rod (C).

Results also showed that *E. limosum* is a Gram-positive rod as described by (Genthner *et al*., 1981) shown in figure 3.5.

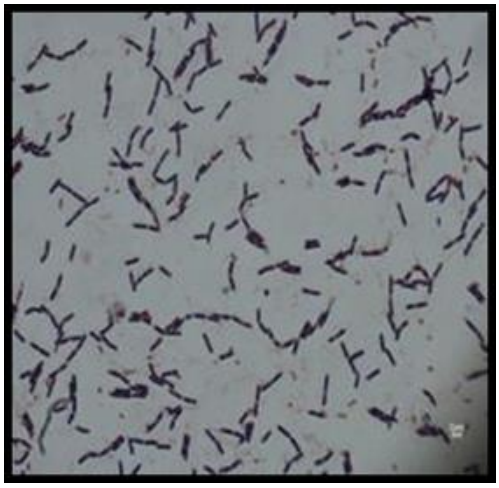

Fig.3.5. Gram staining characteristics of *E. limosum*. Scale bar was set at 2 µm.

3.9.3 Live/dead stain

Cells were stained, using the Live/dead® BacLight TM Bacterial Viability Kit, as described in section 3.6.4, to confirm the viability of the micro-organisms in late exponential phase. The aerobic growth results for the mine isolates showed that *T. scotoductus* SA-01 (Figure 3.6 A), *Geobacillus* sp. A12 (Figure 3.6 B) and *Geobacillus* sp. GE-7 (Figure 3.6 C) were still viable when the late exponential phase was reached. Live cells stain green and dead cells stain red. The stain confirmed comprehensively that the majority of cells were alive.

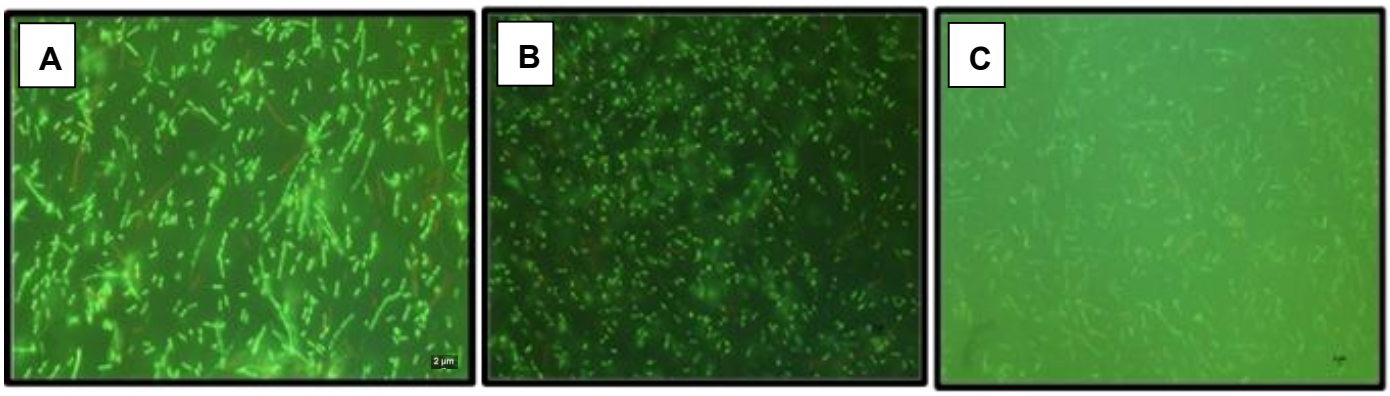

Fig.3.6. Live/dead stain for *T. scotoductus* SA-01 (A), *Geobacillus* sp. A12 (B) and *Geobacillus* sp. GE-7 (C). Scale bars were set at 2 µm.

3.9.4 Anaerobic growth

As described in sections 3.6.1 and 3.6.2, anaerobic growth studies were performed with *T. scotoductus* SA-01, *Geobacillus* sp. A12 and *Geobacillus* sp. GE-7, that all yielded low biomass, except the strict anaerobe, *E. limosum*, that showed good proliferation under these conditions. The growth curve in figure 3.7, indicates that *E. limosum* reached late exponential in approximately 24 hours. This experiment was performed in triplicate.

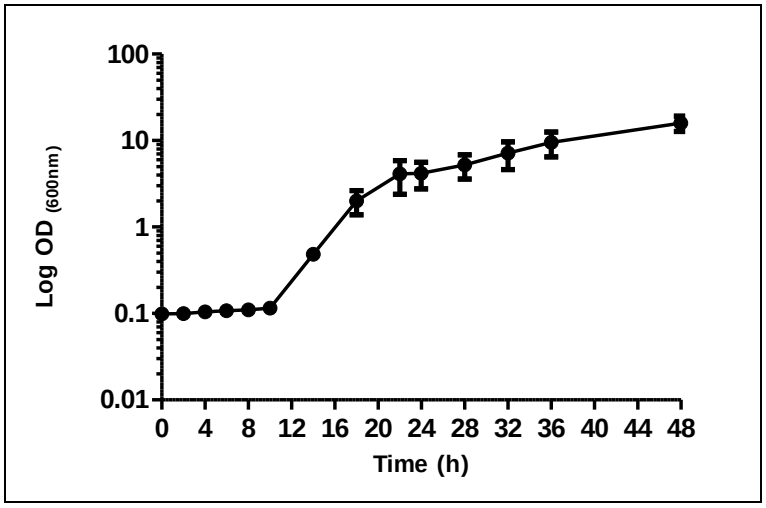

Fig.3.7. Anaerobic growth curve for *E. limosum* where optical density was monitored over time. Standard deviations were seen at 20 to 48 hours.

According to literature, *T. scotoductus* SA-01, *Geobacillus* sp. A12 and *Geobacillus* sp. GE-7 are able to grow anaerobically at very low rates (Kieft *et al*., 1999; DeFlaun *et al*., 2007; Jugdave, 2011). However, due to the low yield in biomass no significant increase in OD_{600} could be detected [Figure 3.8, *T. scotoductus* SA-01 (Blue line), *Geobacillus* sp. GE-7 (Green line) and *Geobacillus* sp. A12 (Red line)]. Therefore, live/dead stains (Figure 3.9), as well as a nitrate reduction analysis were performed (Figure 3.10). Results from the live/dead stain determined that *T. scotoductus* SA-01 (Figure 3.9 A),

Geobacillus sp. A12 (Figure 3.9 B) and *Geobacillus* sp. GE-7 (Figure 3.9 C) after a period of time in anaerobic conditions are still alive. Nitrate reduction during anaerobic growth is shown by nitrite. Balkwill and co-workers (2004) stated that *Thermus* SA-01 is able to use nitrate as terminal electron acceptor. This was confirmed by Gounder and co-workers (2011) indicating that *T. scotoductus* SA-01 contains genes responsible for carrying out denitrification. Figure 3.10 demonstrate' the initial increase in production of nitrite and decrease in production as the period of incubation increases. The comparison of NO_3^- to NO_2^- turnover by *T. scotoductus* SA-01 (Blue line), and *Geobacillus* sp. A12 (Red line) are much higher at stages of growth than *Geobacillus* sp. GE-7 (Green line). The results obtained for *Geobacillus* sp. GE-7 was confirmed by DeFlaun and co-workers (2007), showing that although *Geobacillus* sp. GE-7 was supplemented with nitrate; minimal nitrite was detected in the anaerobic grown cultures. According to Krumholz (2000) and Kieft and co-workers (1999) micro-organisms living in these extreme environments are likely to adapt to gradual growth over a long period of time. Live/dead stain was also performed on *E. limosum*, indicating that the cells were still viable when late exponential phase was reached in the anaerobic growth study (Figure 3.11).

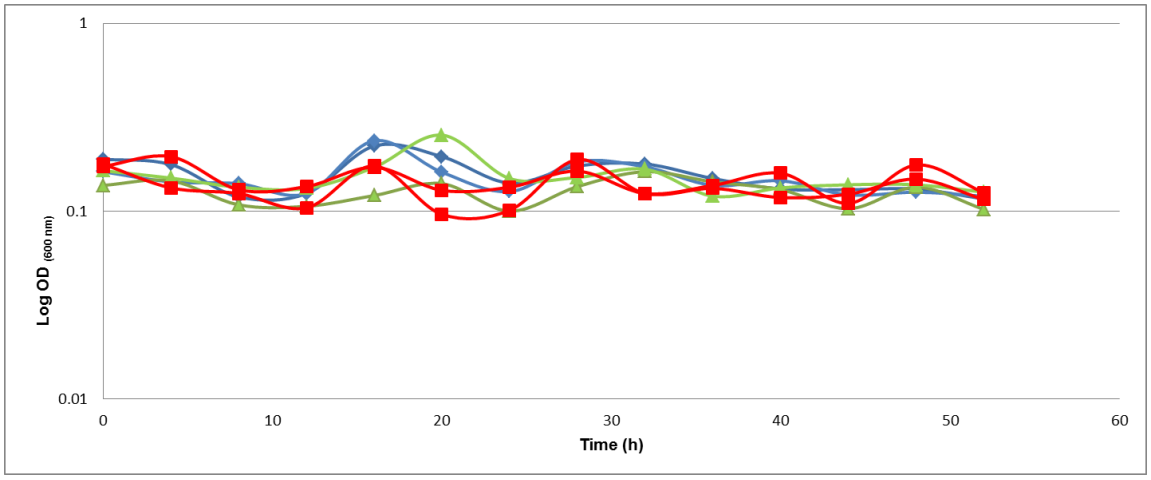

Fig.3.8. Anaerobic growth curves for *T. scotoductus* SA-01 (Blue line), *Geobacillus* sp. GE-7 (Green line) and *Geobacillus* sp. A12 (Red line) where optical density was monitored over time.

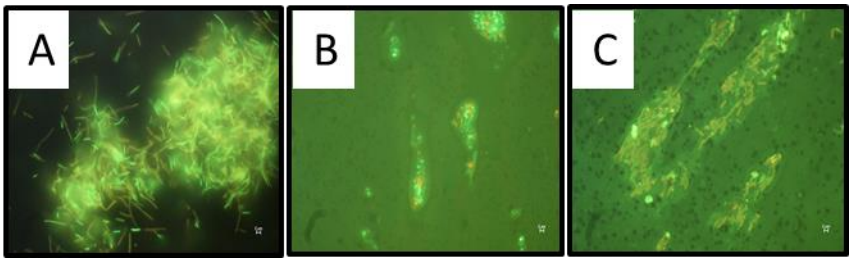

Fig.3.9. Live/dead stains for *T. scotoductus* SA-01 (A), *Geobacillus* sp. A12 (B) and *Geobacillus* sp. GE-7 (C). Scale bars were set at 2 µm.

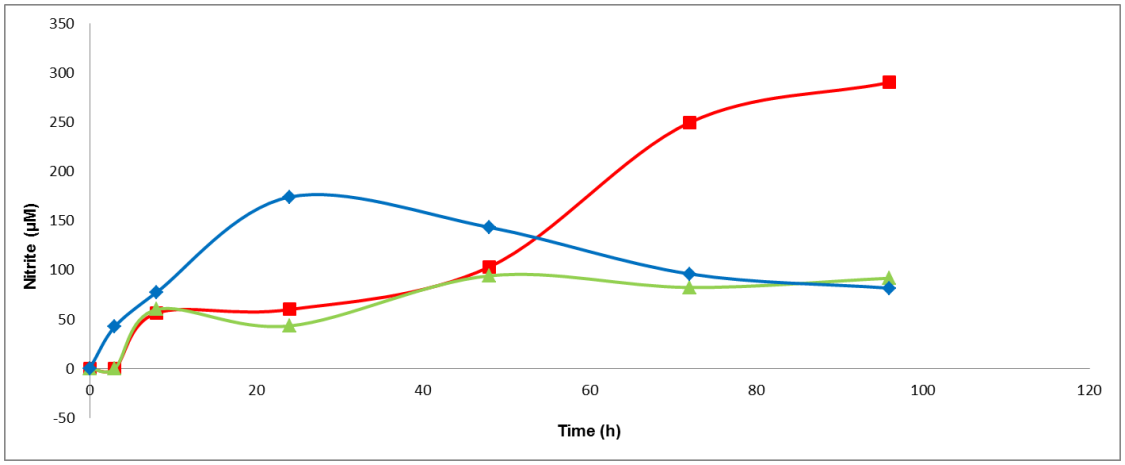

Fig.3.10. Nitrate reduction during anaerobic growth is shown by nitrite. *T. scotoductus* SA-01 (Blue line), *Geobacillus* sp. A12 (Red line) and *Geobacillus* sp. GE-7 (Green line).

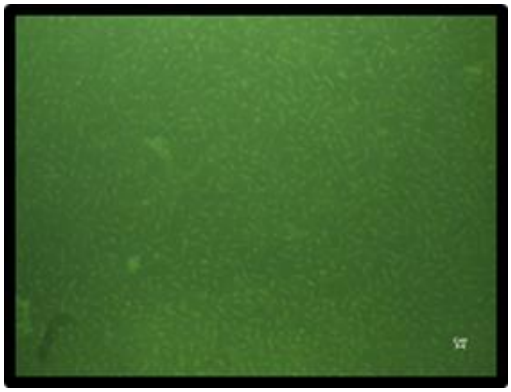

Fig.3.11. Live/dead stain for *E. limosum*. Scale bar was set at 2 µm.

3.9.5 Anoxic growth

Anoxic growth studies were performed as described in sections 3.6.1 and 3.6.2, in duplicate. During anoxic growth, resazurin [0.0003% (v/v)] was added as an oxidation-reduction oxygen indicator as shown by the pink colour (see negative control, figure 3.13 A). Over a period of time, after inoculation, oxygen was slowly reduced, as the biomass of the organisms increased, indicated by the light brown colour in comparison to the control which remained pink, (Figures 3.12 and 3.13 A). This means that in the initial stages of growth, *T. scotoductus* SA-01, *Geobacillus* sp. A12 and *Geobacillus* sp. GE-7 utilized the limited amount of oxygen as final electron acceptor and then in the latter stages started to use potassium nitrate as indicated by the reduction of nitrate (Figure 3.13 B). It took *T. scotoductus* SA-01 (Blue line), approximately 24 hours, with *Geobacillus* sp. GE-7 (Green line), and *Geobacillus* sp. A12 (Red line), approximately 20 hours to reach late exponential phase (Figure 3.12).

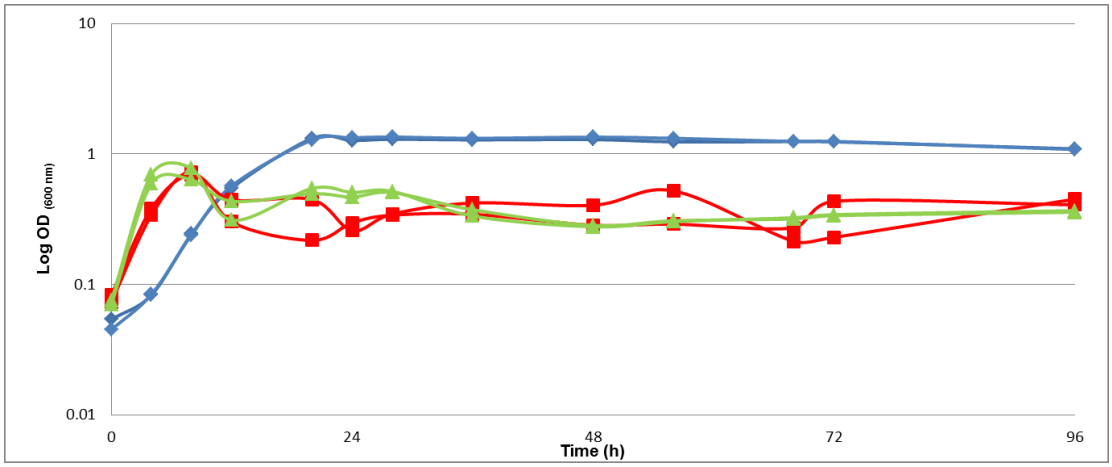

Fig.3.12. Anoxic growth curves for *T. scotoductus SA-01* (Blue line), *Geobacillus* sp. GE-7 (Green line), and *Geobacillus* sp. A12 (Red line) where optical density was monitored over time.

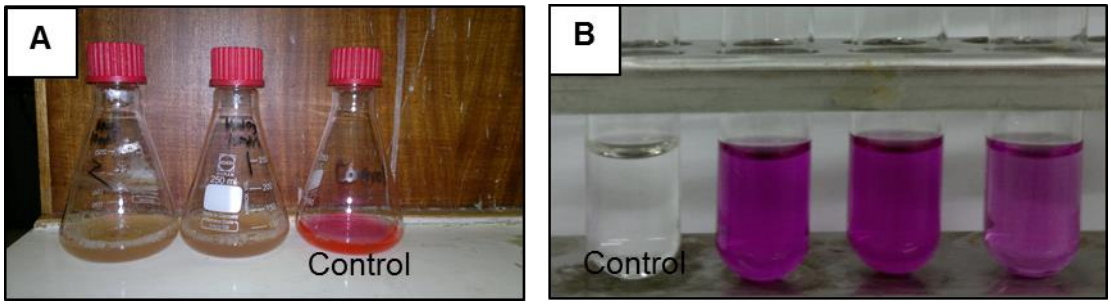

Fig.3.13. Anoxic growth monitored over a period of time. The control remained pink (A). Nitrite detection test, using the Griess Kit, showed pink colour, indicative of nitrite formation (B).

3.9.6 Correlation of cell density, ATP production and OD readings

Optimal growth conditions were used to harvest cells in mid exponential phase. Standard curves, correlating to OD_{600}, cell counts and ATP production are displayed in figure 3.14 A-D. A correlation in the amount of cells (direct count) is shown in relation to the OD_{600}. This specific cell count also displays active and almost linear ATP production (metabolic activity) during active growth. Due to using cells from the exponential phase, a high percentage of ATP production was expected.

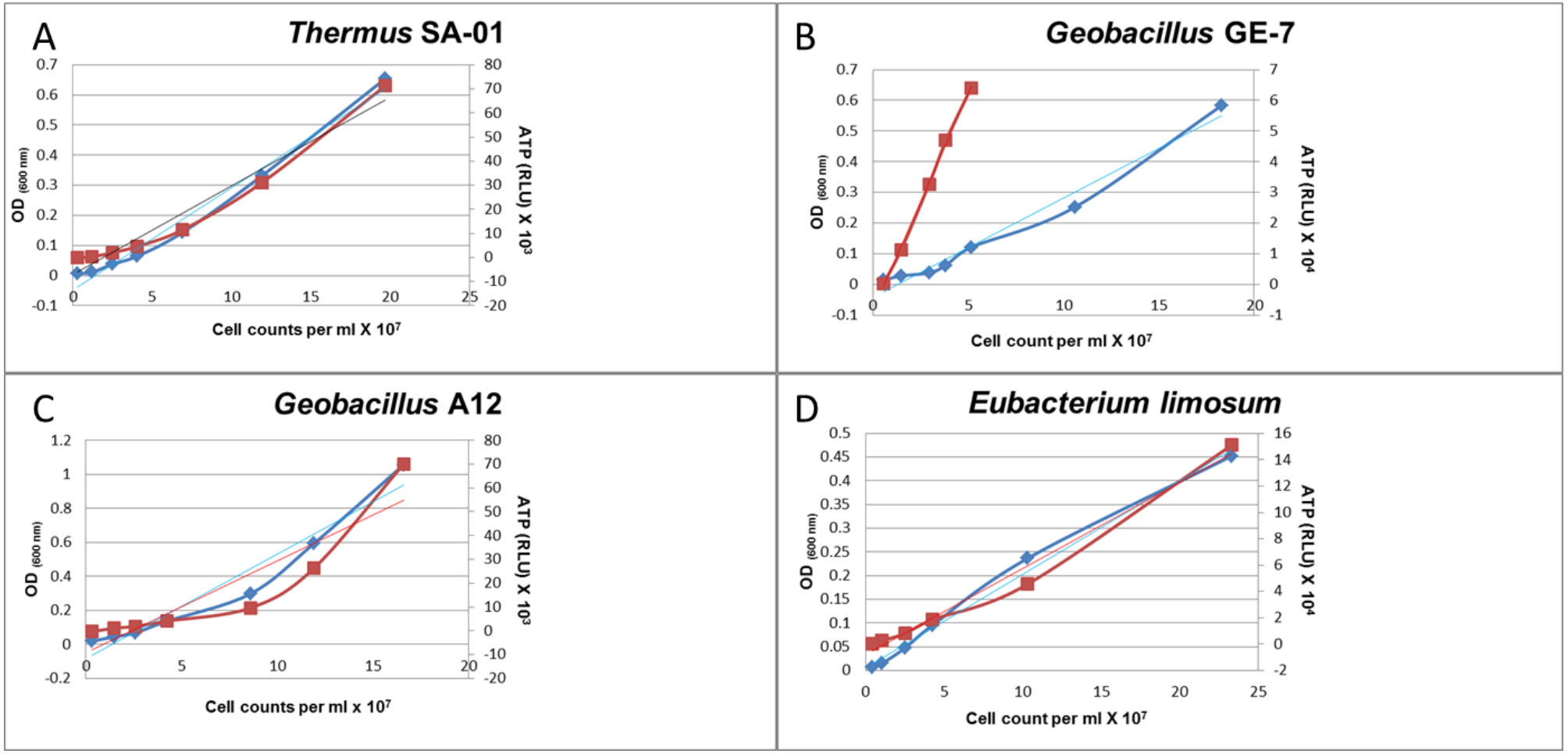

Fig.3.14. Standard curves, indicating the relationship between ATP (RLU), OD_{600} and cell counts per mL. Solids lines define the R^2 value. *T. scotoductus* SA-01 (A) (R^2=0.9806, OD_{600} and cell counts per mL {Blue line} and R^2= 0.9589 cell counts per mL and ATP [RLU] {Red line}), *E. limosum* (D) (R^2=0.9923, OD_{600} and cell counts per mL {Blue line} and R^2= 0.983 cell counts per mL and ATP [RLU] {Red line}), *Geobacillus* sp. GE-7 (B) (R^2=0.9759, OD_{600} and cell counts per mL {Blue line} and R^2= 0.9985 cell counts per mL and ATP [RLU] {Red line}) and *Geobacillus* sp. A12 (C) (R^2=0.9443, OD_{600} and cell counts per mL {Blue line} and R^2= 0.8363 cell counts per mL and ATP [RLU] {Red line}).

3.10 Genomic DNA extraction and amplification of the 16S rRNA genes

Cells were harvested as described in section 3.7 and used for genomic DNA extraction. A high yield of genomic DNA was recovered from all the samples (200-300 ng/µL) as shown in figure 3.15. The genomic DNA was evaluated on a 0.8% (w/v) agarose gel as described in section 3.7.1. The visualization of the DNA showed little to no degradation or shearing, and the A_{260}/A_{280} absorbance ratio indicated a high level of purity.

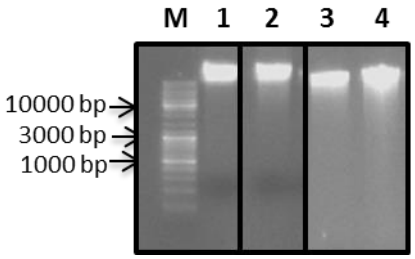

Fig.3.15. Extracted genomic DNA: lane M; GeneRuler™ DNA ladder (Fermentas), lanes 1**:** *T. scotoductus*. SA-01; 2**:** *E. limosum* 3**:** *Geobacillus* sp. A12 4**:** *Geobacillus* sp. GE-7.

The amplification of the 16S rRNA genes were performed as described in section 3.8.1 and amplicons of approximately 1500 bp were obtained as expected for the bacterial 16S rRNA gene amplicons (Figure 3.16). Amplification was done by using a temperature gradient of between 41˚C-52˚C to identify the optimal annealing temperature for 16S rRNA amplification. The PCR products obtained were evaluated on 1% (w/v) agarose gels as shown in figure 3.16 A-D.

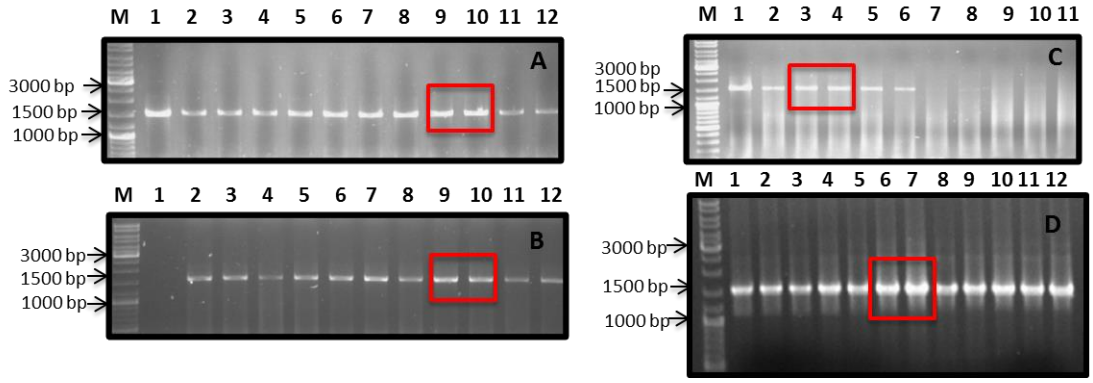

Fig.3.16. Amplification of the 16S rRNA gene amplicons from genomic DNA: Lane M; GeneRuler™ DNA ladder (Fermentas), lanes 1 to 12 are the positive amplified bands of the 16S rRNA genes from *Geobacillus* sp. A12 (A) and *Geobacillus* sp. GE-7 (B) with optimal annealing temperature for both at 49 or 50˚C, indicated in the red box. *T. scotoductus* SA-01 (C) with optimal annealing temperature at 43 or 44˚C, indicated in the red box and *E. limosum* (D) with optimal annealing temperature at 46 or 47˚C, indicated in the red box.

The PCR products were recovered from the agarose gel as described in section 3.8.1. The concentration and purity of the product was measured using a NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE). Purified PCR products were ligated into pGEM®-T Easy and transformed into competent Top 10 *E. coli* cells as described in sections 3.8.3 and 3.8.4. Positive clones (white colonies) were selected from the LB-AIX media plates (IPTG, X-Gal and ampicillin) and grown overnight in 5 mL LB media supplemented with 10 mg/mL ampicillin. If growth was obtained from the overnight culture, plasmid extractions were carried out as described in section 3.8.4.

3.10.1 Molecular identification of bacterial strains

Restriction digest was performed according to section 3.8.5 on the colonies retrieved in section 3.10. The backbone of the vector (~3000 bp) was released with restriction

enzymes *Nco*I and *Sal*I. Sequences retrieved from NCBI for the four selected micro-organisms showed that the restriction enzymes *Nco*I and *Sal*I do not digest the 16S rRNA sequences, thus releasing the 1500 bp insert. A 1% (w/v) agarose gel was run to visualize the digested products shown in figure 3.17. The confirmed clones were then used in subsequent sequencing reactions for identification purposes as described in sections 3.8.6 and 3.8.7.

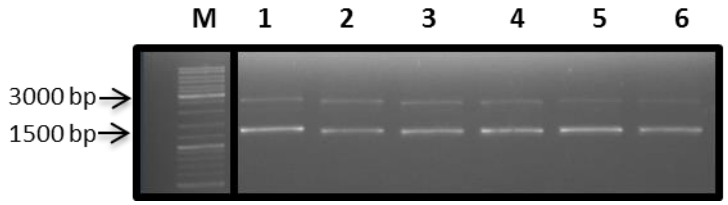

Fig.3.17. Restriction digest of *Geobacillus* sp. A12 (lane 1), *Geobacillus* sp. GE-7 (lane 2), *T. scotoductus* SA-01 (lanes 3 and 4) and *E. limosum* (lanes 5 and 6) in pGEM®-T Easy. pGEM®-T Easy indicated by the 3000 bp fragment on the gel and the 1500 bp indicating the product of interest. Lane M; GeneRuler™ DNA ladder (Fermentas).

3.10.2 DNA sequencing results

The cloned 16S rRNA gene PCR products were sequenced using the Big Dye terminator v.3.1 Cycle sequencing kit and purified as described in sections 3.8.6 and 3.8.7. The sequences were assembled and evaluated (sequence results presented in supplement A, section 3.12). The 16S rRNA genes present in the genome of the bacterial spp. were subjected to BLAST analysis against the NCBI database to determine the identity of the organisms (Table 3.4).

Table 3.4: Results obtained after BLAST analysis of the 16S rRNA gene sequences of *E. limosum*, *T. scotoductus* SA-01, *Geobacillus* sp. GE-7 and *Geobacillus* sp. A12.

Accession	Description	E value	Max ident
JQ085755.1	*Eubacterium limosum* strain ZL2 16S ribosomal RNA, partial sequence	0.0	99%
NR_074428.1	*Thermus scotoductus* SA-01 strain SA-01 16S ribosomal RNA, complete sequence	0.0	99%
AB034836.2	*Geobacillus thermoleovorans* gene for 16S rRNA, partial sequence	0.0	99%
KC420686.1	*Geobacillus kaustophilus* strain S12-1-1 16S ribosomal RNA gene, partial sequence	0.0	99%
JX298768.1	*Geobacillus lituanicus* strain GMQ1 16S ribosomal RNA gene, partial sequence	0.0	99%
KC252981.1	*Geobacillus thermoparaffinivorans* strain Ba7 16S ribosomal RNA gene, partial sequence	0.0	99%

Geobacillus sp. GE-7 and *Geobacillus* sp. A12 both belong to the *Geobacillus* genus. The results confirmed initial identification that correlated well with published results (DeFlaun *et al*., 2007). However; the taxonomic characterization of this genus using a single molecular technique is not considered to be significant. More techniques to successfully complete species description are used such as Ribotyping or housekeeping genes.

3.11 Conclusions

The identity of the three subsurface micro-organisms and a commercial strain were confirmed using molecular identification. This is an important aspect, as the genome sequences available for these micro-organisms, can be used to confirm availability of certain metabolic pathways. The identity of *E. limosum* was confirmed and has been proven to grow anaerobically. The three subsurface strains *T. scotoductus* SA-01, *Geobacillus* sp. A12, and *Geobacillus* sp. GE-7 displayed good aerobic growth rates, but were also able to sustain themselves metabolically under anaerobic conditions as seen by the live/dead stain and the reduction of nitrate over time. This was also confirmed with anoxic growth experiments where a limited amount of oxygen was available at the initial stages of growth and over a period of time, the oxygen was depleted (medium turned brown/yellow when compared to the control). Nitrite reduction was also seen in both anoxic and anaerobic growth characteristics. The cells remained viable and metabolically active even in late exponential phase. The mid exponential, actively growing cells, showed high ATP production that is encouraging when metabolic capabilities are studied.

3.12 Supplement A

3.12.1 Sequence data of 16S rRNA genes for selected micro-organisms

The 16S rRNA gene sequence of *E. limosum* used for identification.

GGGCCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGCAACCCTGACGCAG
CANTACCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTGTTATTGGGGAAGAAGA
ATGACGGTACCCAATGAGGAAGTCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGGGACAAGCGTTGTCCGGAATGACTGGGCGTAAAGGGCGCGTAGGCGGTCTATTAA
GTCTGATGTGAAAGGTACCGGCTCAACCGGTGAAGTGCATTGGAAACTGGTAGACTTGA
GTATTGGAGAGGCAAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGA
ACACCAGTGGCGAAGGCGGCTTGCTGGACAAATACTGACGCTGAGGTGCGAAAGCGTG
GGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCTAGGTGT
TGGGGAAACTCAGTGCCGCAGTTAACACAATAAGCATTCCGCCTGGGGAGTACGACCGC
AAGGTTGAAACTCAAAGGAATTGACGG

The 16S rRNA gene sequence of *T. scotoductus* SA-01 used for identification.

TCGATTGGTTACCTTGTTACTACTTCGCCCCAGTCACGAGCCCTACCCTCGGCGCCTGCC
CTAAGGCTCCCGGCGACTTCGGGTAGAACCCGCTCCCATGGCGTGACGGGCGGTGTGT
ACAAGGCCCGGGAACGTATTCACCGCGGCATGGCTGATCCGCGATTACTAGCGATTCCG
GCTTCATGGGGTCGGGTTGCAGACCCCAATCCGAACTACGCCCACCTTTTTGCGATTCG
CTCCCCATCACTGGGTCGCCTCGCTCTGTAGTGGGCATTGTAGCACGTGTGTCGCCCAG
GCCGTAAGGGCCATGATGACCAGACGTCGTCCCCGCCTTCCTCCTGCTTTCGCAGGCAG
TCCCCTTAGAGTGCCCGGCCTATCCCGCTGGCAACTAAGGGCAGGGGTTGCGCTCGTTG
CGGGACTTAACCCAACATCTCACGACACGAGCTGACGACGGCCATGCAGCACCTGTGCT
AGGGCTCCCTCGCGGGTACCCCAGGCTTTCACCTAGGTCCCCTAGCATGTCAAGGCCTG
GTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCC
GTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGCGCGCTTAACGCG
TTGGCTTCGGCCCCCAGGTAAACCCCAAAGACCTAGCGCGCATCGTTTAGGGCGTGGAC
TACCCGGGTATCTAATCCGGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGAAGTGG
ACCAGGTGGCTGCCTTCGCCATCGGCGTTCCTCCCGGTATCTGCGCATTTCACCGCTAC
TTCGGGAATTCCACCACCCTCTCCCACCCTCTAGCCTGAGCGTATCCCACGCTCCTCCAC
GGTTGAGCCGCGGCCCTTTCACATGGGACGCCCCAGGCCGCCCTACACGCCCTTTACG
CCCAGTAAATCCGGG

The 16S rRNA gene sequence of *Geobacillus* sp. GE-7 used for identification.

GTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCC
GGCTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGAGCGGCTTTTTGGGATTC
GCTCCCCCTCGCGGGTTCGCAGCCCTTTGTACCGCCCATTGTAGCACGTGTGTAGCCCA
GGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGACTTGTCGCCGGCA
GTCCCTCTAGAGTGCCCACCTTCGTGCTGGCAACTAGAGGCGAGGGTTGCGCTCGTTGC
GGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACC
CTGTCCCCCCGAAGGGGGAACGCCCAATCTCTTGGGTTGTCAGGGGATGTCAAGACCT
GGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCC
CCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAGTGCTTATCG
CGTTAGCTGCAGCACTAAAGGGTGTGACCCCTCTAACACTTAGCACTCATCGTTTACGGC
GTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGT
TGCAGGCCAGAGAGCCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACC
GCTACACGTGGAATTCCGCTCTCCTCTCCTGCACTCAAGTCCCCCAGTTTCCAATGACCC
TCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGGRACCGCCTGCGCGCGCTTTA
CGCCCAATAATTCCGGACAACGCTCGCCCCCTACGTATTACCGCGGCTGCTGGCACGTA
GTTAGCCGGGGCTTYCTCGTGAGGTACCGTCACCGCGCCGCCCTCTTCGAACGGCGCT
CCTTCGTCCCTCACAACAGAGCTTTACGACCCGAAGGCCTTCTTCGCTCACGCGGCGTC
GCTCC

The 16S rRNA gene sequence of *Geobacillus* sp. A12 used for identification.

GTGCCAGCAGCCGCGGTAATACGTAGGGGGCGAGCGTTGTCCGGAATTATTGGGCGTAA
AGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCYACGGCTCAACCGTGGAGG
GTCATTGGAAACTGGGGGACTTGAGTGCAGGAGAGGAGAGCGGAATTCCACGTGTAGC
GGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGCCTGC
AACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGGTCACACCCTTTAGTGCTGCAGCTAA
CGCGATAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTA
CCAGGTCTTGACATCCCCTGACAACCCAAGAGATTGGGCGTTCCCCCTTCGGGGGGACA
GGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTCGCCTCTAGTTGCCAGCACGAAGGTGGGCACTCTAGAGGG
ACTGCCGGCGACAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATG
ACCTGGGCTACACACGTGCTACAATGGGCGGTACAAAGGGCTGCGAACCCGCGAGGGG
GAGCGAATCCCAAAAAGCCGCTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGA
AGCCGGAATCGCTAGTAATCGCGGATCAGCATGCC

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CHAPTER 4

CHAPTER 4

PRESSURE STUDIES

4. Introduction

Micro-organisms, specifically Bacteria, are continuously being discovered living under extreme environmental conditions, which are previously thought to be unable to sustain life. Recent discoveries has proven that bacteria can survive at gigapascal pressures and at temperatures as high as 122˚C and as low as –20˚C (DeFlaun *et al*., 2007). Almost all major groups of prokaryotes are able to use CO_2 as their carbon source, meaning growing autotrophically, and providing continuous supply of organic carbon for heterotrophs. In the subsurface is a world exposed to extremely high pressures. Micro-organisms living in the subsurface have several unique features to adapt to such an extreme environment. Barophiles are micro-organisms that grow optimally at high pressures >400 bar or grow better at pressures higher than atmospheric pressure whereas barotolerant bacteria grow optimally at pressure <400 bar and can grow well at atmospheric pressure (Horikoshi, 1998; Margesin & Schinner, 2001). Carbon capture and storage, associated with terrestrial storage at depths greater than 600 m to 1000 m, are associated with pressure and temperature. Depths greater than 600 m with pressure greater than 73 bar and temperatures higher than 31˚C, causes CO_2 to exist in its supercritical state as described in chapter 1 (Gupta, 2006; Holloway 2007). CO_2 in its supercritical state has the high-density characteristics of a liquid but behaves like a gas that fills up the available pore space within the medium (Thompson *et al*., 2012). Thus, barophiles are great

candidates for CCS applications. The Calvin cycle represents the most important autotrophic carbon fixation pathway. This cycle is restricted to organisms that yield high-energy from chemotrophic or phototrophic organisms. Micro-organisms that live in extreme environments such as high temperatures, anaerobic or acidic conditions, generally utilize different CO_2 fixation pathways (Atomi, 2002; Hügler *et al*., 2005).

4.1 Autotrophic pathways

Since 2011, six autotrophic carbon fixation pathways have been described. The Calvin cycle, the reductive citric acid cycle (rTCA), the reductive acetyl-CoA pathway (rAcCoA), the 3-hydroxypropionate cycle, the 3-hydroxypropionate/4-hydroxybutyrate cycle and the dicarboxylate/4-hydroxybutyrate cycle. The reductive citric acid cycle, otherwise known as the Arnon-Buchanan cycle was proposed in 1966 (Evans *et al*., 1966). It has been described in anaerobic and microaerobic bacteria, where the oxidative citric acid cycle runs in reverse. The reductive acetyl-CoA pathway, otherwise known as the Wood-Ljungdahl pathway, was proposed in 1965 and is found in strictly anaerobic bacteria and archaea (Ljungdahl, 1986; Wood, 1991). The 3-hydroxypropionate cycle was proposed in 2002 and is only known in green non-sulphur bacteria that are especially oxygen sensitive (Herter *et al*., 2002; Zarzycki *et al*., 2009). The last two pathways were recently described in the (hyper) thermophilic, autotrophic Crenarchaeota. The two pathways are restricted to this group of Archaea. The 3-hydroxypropionate/4-hydroxybutyrate cycle, proposed in 2007, has been found in aerobic Archaea (Berg *et al*., 2007), while the dicarboxylate/4-hydroxybutyrate cycle, proposed in 2008, has been found in anaerobic Archaea (Huber *et al.,* 2008). The Calvin-Benson-Bassham cycle is mostly used by autotrophic organisms for CO_2

assimilation but the process can be limited by the low catalysis rate of the Rubisco enzyme (ribulose-1,5-bisphosphate carboxylase/oxygenase) (Bar-Even *et al*., 2010). Depending on the carbon fixation pathways utilized, certain amounts of ATP molecules are involved in hydrolysis, which will then decrease the energetic efficiency and increase the energetic cost (Berg *et al*., 2010; Berg, 2011; Hügler & Sievert, 2011).

4.1.1 Calvin cycle (rPP)

Carbon fixation is the reduction of inorganic CO_2 to organic compounds by living organisms. (Bar-Even *et al*., 2010). The Calvin cycle is also known as the reductive pentose phosphate (rPP) cycle or the Calvin-Benson-Bassham cycle. The enzyme that fixes CO_2 in this cycle is the ribulose 1,5-bisphosphate carboxylase/oxygenase otherwise known as Rubisco (Flachmann *et al*., 1997). A number of prokaryotes and photosynthetic eukaryotic organisms were found to rely on the Calvin cycle for CO_2 fixation, and many of them have been shown to contain the enzyme Rubisco. For example, purple non-sulphur bacteria (*Rhodobacter*), purple sulphur bacteria (*Chromatium*), cyanobacteria *(Anabaena*), hydrogen bacteria (*Hydrogenovibrio*) and other chemoautotrophs (*Thiobacillus*) have been shown to utilize the Calvin cycle (Atomi, 2002). Thirteen enzymatic reactions are required for the Calvin cycle. There are three distinct stages in the Calvin cycle shown in figure 4.1. The first stage is known to be carbon fixation and the twelve reactions are used to regenerate Rubisco. Stage two is known as the carbon reduction reaction, each molecule of 3-phosphoglycerate is phosphorylated by using the ATP produced during the light reactions. 3-phosphoglycerate is then reduced to glyceraldehydes 3-phosphate

(GAP) using NADPH that was also produced during the light reactions. The third stage, known as regeneration, is where the starter molecule is regenerated. Glyceraldehyde-3-phosphate goes through a series of reactions where it leaves the cycle to be further synthesized into sugars and other macromolecules needed for cellular metabolism. Here five carbon molecules of ribulose 1,5-bisphosphate are produced and the cycle begins again (Atomi, 2002; Fuchs, 2011).

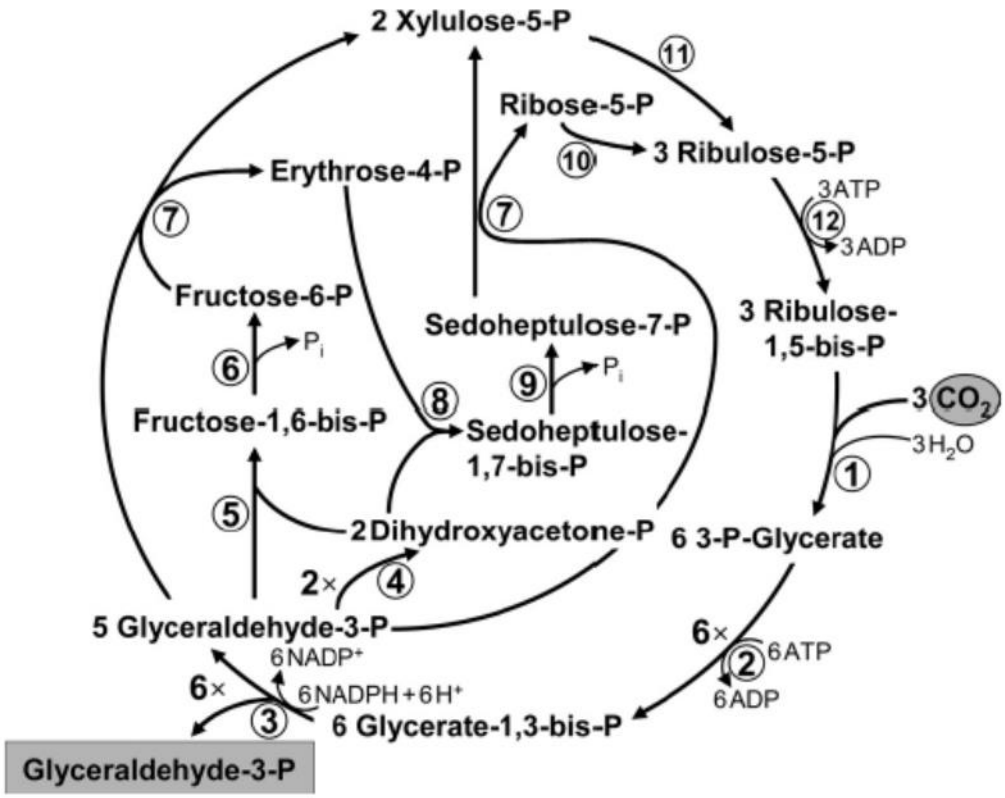

Fig.4.1. Calvin-Benson-Bassham cycle (Taken from Berg, 2011).

There are three enzymes that can be considered unique to the Calvin cycle: Rubisco, phosphoribulokinase (PRK), and sedoheptulose bisphosphatase (SBPase). The other enzymes are shared with the gluconeogenesis pathway and the pentose phosphate cycle (Shively *et al*., 1998). This process is very energy consuming (Fuchs, 2011).

4.1.2 Reductive tricarboxylic acid cycle (rTCA) and Reductive Acetyl Co-enzyme A cycle (rAcCoA)

Organisms that operate the rTCA cycle of the rAcCoA pathways are known to survive in high CO_2 habitats or operate a carbon concentrating mechanism. These organisms are generally anaerobic and energy restricted when compared to aerobes which limits their available energy for investment in carbon fixation. The reductive tricarboxylic acid cycle (rTCA) is an alternative pathway for fixing CO_2 by reversing the TCA cycle shown in figure 4.2. The rTCA cycle is one of the simplest cycles where only four enzymes are used. Therefore, instead of breaking down acetyl-CoA with the release of two CO_2 molecules, acetyl-CoA is synthesized by two CO_2 molecules. The product produced by this simple cycle is glyoxylate and it is converted to GA3P by the bacterial-like glycerate pathway. However, the ATP citrate lyase enzyme plays a key role in the rTCA cycle because it cleaves citrate (which has six carbons) into oxaloacetate (four carbons) and acetyl CoA (which has two carbons) (Hügler *et al*., 2005). The reductive acetyl co-enzyme A cycle describes autotrophic production of acetyl-CoA from two CO_2 molecules shown in figure 4.3. The rTCA cycle or the rAcCOA pathways are more ATP-efficient than the rPP cycle (Bar-Even *et al*., 2010). The Calvin cycle, the rTCA cycle, and the 3-hydroxypropionate cycle are present in both photo- and chemoautotrophic micro-organisms, whereas the Acetyl-CoA pathway is confined to chemoautotrophs (Atomi, 2002; Hügler & Sievert, 2011). The 3-hydroxypropionate cycle is especially oxygen sensitive and energy intensive. The 3-hydroxypropionate/4-hydroxybutyrate cycle, found in aerobic Archaea' and dicarboxylate/4-hydroxybutyrate cycle, found in anaerobic Archaea' were recently described in the (hyper) thermophilic, autotrophic

Crenarchaeota (Berg *et al*., 2007), but these additional cycles are not discussed in detail.

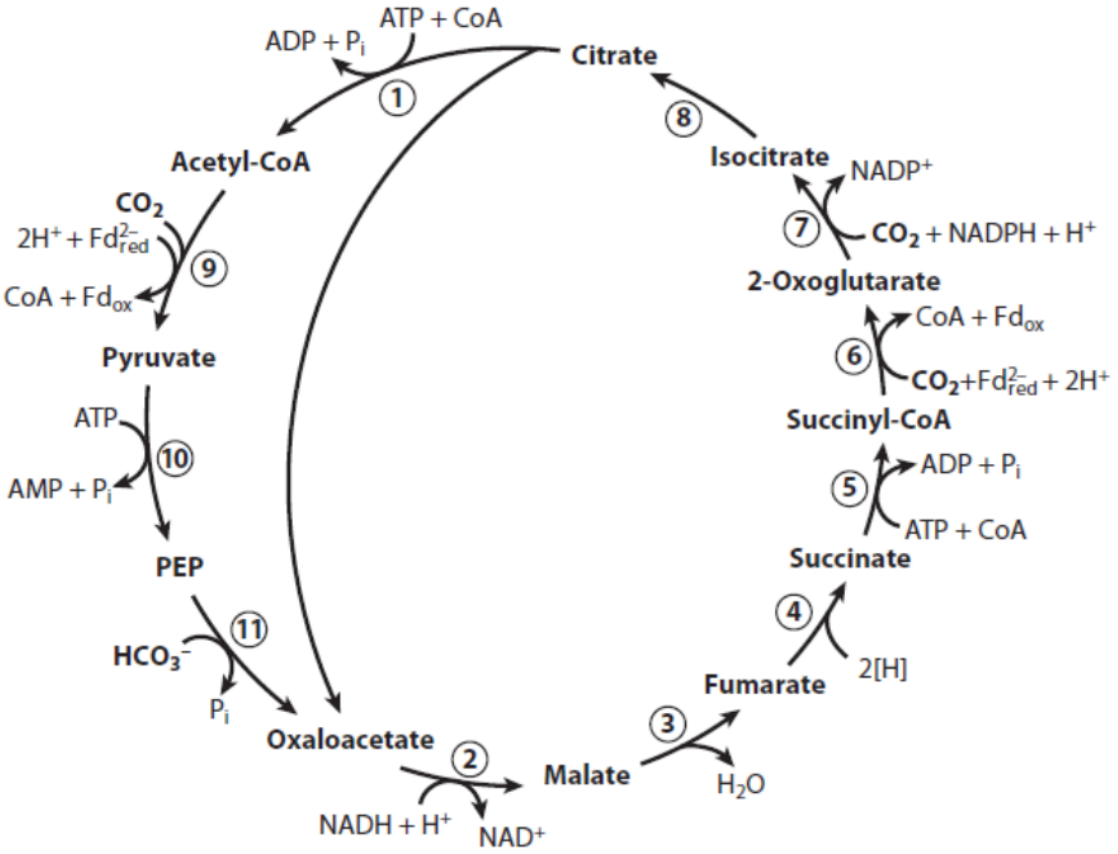

Fig.4.2. Reductive tricarboxylic acid cycle (Taken from Fuchs, 2011).

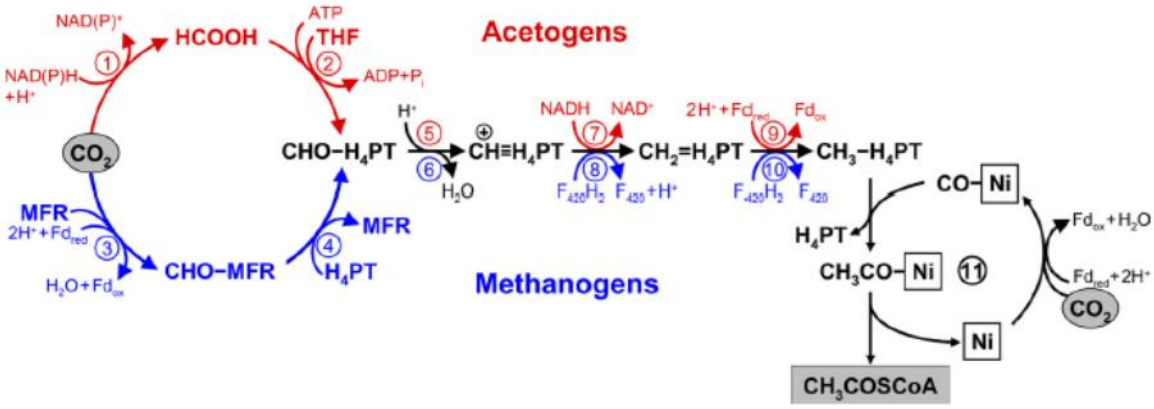

Fig.4.3. Reductive acetyl-CoA cycle (Taken from Berg, 2011).

4.2 Supercritical CO_2 effect on cells

Figure 4.4 shows how CO_2 affects the cell membrane. A CO_2 molecule, which is hydrophobic and liposoluble, can affect microbial processes. This molecule freely transits cell membranes and alters the properties of the membrane and affect a variety of the intracellular functions (Darani & Mozafari, 2010; Wu *et al*., 2010). As CO_2 accumulates in the cytoplasm of the cell, it lowers the pH and causes disorder of the lipid chains in the bilayer membrane which causes the viscosity of the membrane to reduce, resulting in cytoplasm leakage as well as a change in the intracellular salt concentrations. However, conformation of critical proteins that are essential for regulating functions, such as active transport of amino acids, ions and peptides are changed, affecting glycolysis and proton translocation (Oulé *et al*., 2006).

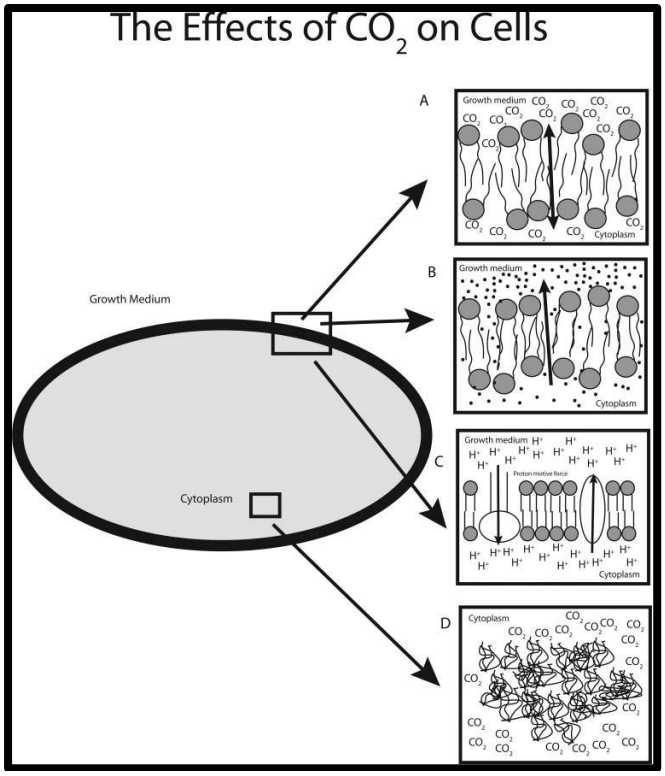

Fig.4.4. Schematic representation of how CO_2 affects the bacterial cells under high pressure. A). When CO_2 is added it alters the membrane fluidity. B) The intracellular salt concentration changes due to the altered membrane fluidity. C) CO_2 increases the acidity in the medium which interferes with the proton motive force. D) Due to the acidity the cell's cytoplasm denatures and deactivates the intracellular proteins (Santillan *et al*., 2013).

Research has been done on microbes that were exposed to SC-CO_2 in a reactor and observed through an electron microscope, with cells stained using the live/dead staining kit. CO_2 in its supercritical state possesses a significant diffusion coefficient and low viscosity. Microbial growth is inhibited by CO_2 in a reversible way under atmospheric pressure. As the pressure increases, CO_2 affects the micro-organisms and becomes irreversible. Pressurized CO_2 appears to effect microbicidal activity in several parameters, such as the type of microorganism, medium, time of exposure, temperature as well as with a reduction in the pH of the medium (Ballestra & Cuq, 1998). Oulé and co-workers (2006) showed that supercritical CO_2 has a powerful capacity of diffusion in the cells, producing a rapid overwhelming effect on the cell,

causing cellular death. The inactivation process in liquid or vapour CO_2 involves two distinct steps (Lin *et al*., 1994). Firstly, cellular stress is induced by the relatively slow diffusion of CO_2 across the membrane that provokes a weakening of the cellular envelope, which then leads to rupture of lipid-protein interactions. Once the CO_2 is in the lipid phase of the membrane, it causes a drop in membrane viscosity, because of the phospholipid solubilization (Sears & Eisenberg, 1961). However, the effect is reversible because when the CO_2 is removed from the cells, they synthesize new proteins to repair the damage and start growing again. Secondly, the inactivation phase leading to cell death by penetrating into the cell, causing irreversible damage. However, the efficiency of vapour CO_2 may vary depending on the micro-organism. Popper & Knorr (1990) have reported that sizes and shapes of the bacteria were factors in resistance to pressurized CO_2. A Gram-positive bacterium seem to be more resistant to pressurized CO_2 because of its thick layer of peptidoglycan and slows down CO_2 penetration into the cell when compared to Gram-negative bacteria because of the changes due to phospholipid solubilization by the CO_2 in the two membranes (Oulé *et al*., 2010).

The difference between thermophiles and mesophiles are mainly in their protein and lipid structure, and composition of their membranes. Thermophiles have long phospholipids, saturated, branched fatty acids and have an enzyme system that has adapted to high temperatures when compared to mesophile membranes with shorter, less-saturated, less-branched fatty acids. The presence of multiple carbon-carbon double bonds in unsaturated fatty acids creates more free space between the phosphodiglycerides. These spaces allow the rapid diffusion and accumulation of CO_2 in the membrane and cytoplasm of cells (Darani & Mozafari, 2010; Oulé *et al*.,

2010).

Aerobic micro-organisms and facultative anaerobes utilize O_2 for energy-producing reactions. The need for O_2 seems to be a factor of resistance to the effect of pressurized CO_2. Oxygen is toxic to anaerobes while microaerophiles are able to withstand small concentrations of O_2. However, the vulnerability of these micro-organisms may be linked to the energy availability. The majority of the energy produced during cellular respiration and the available nutrients in the medium are necessary for the survival of aerobes and facultative anaerobes (Pelczar *et al*., 1993). During respiration, energy produced is stored in the form of ATP. The final electron acceptor is O_2 and the final electron acceptor in anaerobic respiration could be CO_2. However, CO_2 is a decoupler of oxidative phosphorylation, therefore, the absence of O_2 and the presence of CO_2 may delay the production of energy in strict aerobes and facultative anaerobes. CO_2 is a hydrophobic molecule that is able to diffuse in membranes and upset protein-lipid interactions, similar to ethanol which is an amphoteric molecule that is able to attach itself to membrane proteins. This contributes to membrane destabilization. However, influencing the physical properties of liquid or supercritical CO_2, creates free radicals in their metabolism which can affect lipids, nucleic acids and proteins (Marquis & Thom, 1992; Oulé *et al*., 2010). CO_2 can penetrate through the cytoderm and interact with the intracellular elements or increase the acidity of the equilibrium aqueous phase through the formation of carbonic acid which is a chemical agent that inactivates the cell. Thus, under high pressures of CO_2 the cells swell and rupture during decompression (Shadrin *et al*., 2009). Oulé and co-workers (2010) described that the microbicidal activity of CO_2 is based on pressure, temperature and the type of micro-organisms and the resistance

of the bacteria towards SC-CO_2 depends on their morphology, structure and physiology.

4.3 Aims of this chapter

The main aims of this chapter were to:

- Evaluate if selected micro-organisms' the Gram-negative *Thermus scotoductus* SA-01, and the Gram-positive *Geobacillus thermoleovorans* GE-7 and *Geobacillus thermoparaffinivorans* A12, and *Eubacterium limosum* are capable of survival under increasing pressure and remain active under supercritical CO_2 to explore carbon cycling.
- Evaluate if the CO_2 concentration increases with increasing pressure to supercritical CO_2 and to explore if this environment effects the survival of the selected micro-organisms.
- Detect if there is any cycling of the CO_2 possible under these conditions.

4.4 Materials and methods

4.4.1 Low pressure studies

Anoxic and anaerobic media were prepared in Balch tubes, as described in section 3.6.1 table 1 and autoclaved. No pressurized tubes were autoclaved as safety precaution. Micro-organisms were revived by inoculating 5 mL of glycerol stock from the -80˚C into a 45 mL medium in a shake flask and grown to its mid exponential phase at an OD_{600} of approximately 0.8. A 50% (v/v) inoculum from the growing cells

was used for the low and high pressure studies in this chapter. The Balch tubes containing 50% (v/v) of inoculum and media were pressurized to 202.6 kPa (2 bar), using a gas mixture of 20% CO_2 and 80% H_2 (Genthner *et al*., 1981). By introducing a gas mixture, a 2 bar environment was created as described in section 4.4.2. The Balch tubes, inoculated with *E. limosum* were then incubated at 34˚C and the deep mine micro-organisms at 55˚C. OD readings for the low pressure studies were recorded, every two hours for *E. limosum* and every six hours for the deep mine micro-organisms, but with a variation using photometer at 605 nm (PhotoLab® S6). These experiments were done in triplicate.

4.4.2 Calculations for gas concentrations

Table 4.1: Calculations for different ratios of CO_2 and H_2 gas concentrations.

Ratio	20%:80%	50%:50%	80%:20%	100%
CO_2	0.4 bar	1 bar	1.6 bar	2 bar
H_2	1.6 bar	1 bar	0.4 bar	0 bar

Regulators were set at different pressures to introduce the different gasses. For example, 2 bar at 20% CO_2 = (0.2 fraction of the total), meaning 2 bar x 0.2 fraction = 0.4 bar shown in figure 4.5 B. The bottle was gassed, without venting to an overpressure of 0.4 bar of CO_2. 2 bar at 80% H_2 = (0.8 fraction of the total), meaning 0.8 x 2 bar = 1.6 bar. The bottle was gassed, without venting, to another 1.6 bar. Thus, 20% CO_2 and 80% H_2 equals to 2 bar, shown in figure 4.5 C. The solubility, availability of CO_2 at 2 bar at room temperature will be approximately 1.216 mmoles (Carroll *et al*., 1991). To calculate the solubility of 100% CO_2, when introduced into a 20 mL Balch tube, equation 4.1 was used to calculate the amount of CO_2 mmoles

present in the tube at room temperature. The calculation was based on assumption that the Balch tube contains 5 mL medium and 15 mL headspace. An internal standard (1% Argon) was introduced into the tubes with 100% CO_2 for analytical calculations. The ratio between the gasses will be 99:1, which is therefore, 1.188:0.012 mmoles of CO_2:Ar.

[PV=nRT] (Equation 4.1)

(P) = pressure

(V) = volume

(n) = moles

(R) = is the ideal (8.314 $J \cdot K^{-1} \cdot mol^{-1}$)

(T)= temperature

High pressure tubing was connected on the individual gas tanks so that each had a regulator. The tubing was then connected to a syringe with a sterile syringe filter (0.22 µM) and a needle attached to it. All the loose ends were clamped tight to prevent tubes becoming over pressurized and care was taken when pressure was introduced into the Balch tubes or serum vials, by pressurizing in a bullet proof box, using cut and fire resistance gloves to prevent unnecessary injuries or breakage of glass wear (Figure 4.5 A and D).

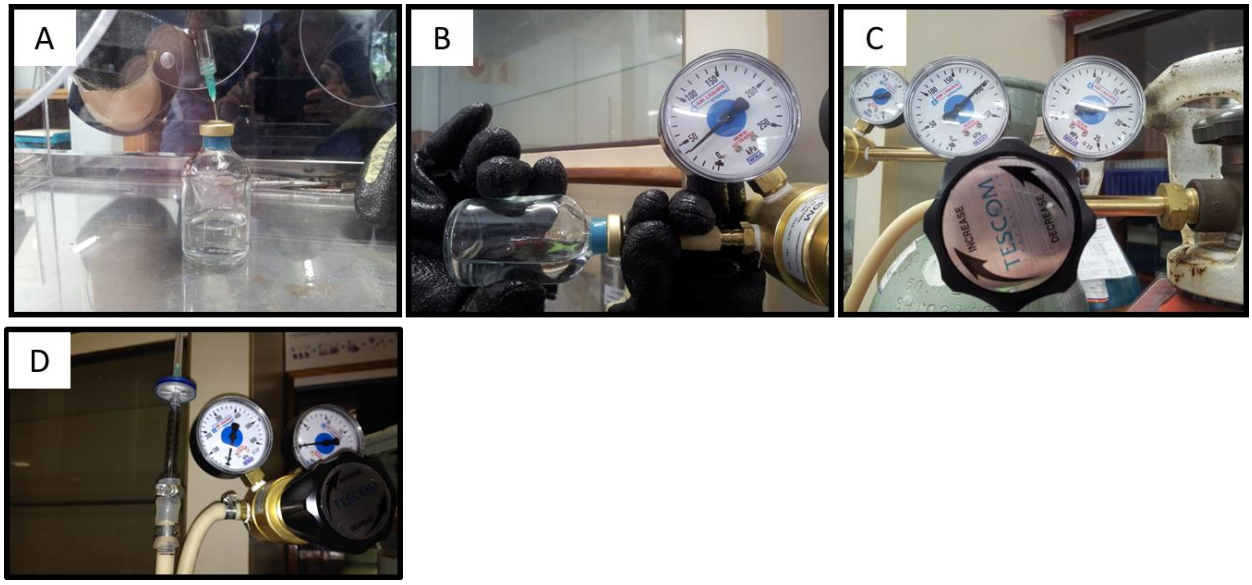

Fig.4.5. Pressuring a gas mixture of 20% CO_2 and 80% H_2 that equals to 2 bar.

4.4.3 Introducing different gas components

The CO_2 tank was turned on and the regulator opened to allow flow. Gas compositions were as described in section 4.4.2. The line was flushed for 30-45 seconds and the needle was inserted with gas flowing into the tube. The tube was turned upside down for the gas to equilibrate between the delivery hose and the tube. Bubbles of gas entering the inverted tube through the media were seen, insuring that gas was delivered. The tube was then turned right-side up and the needle was pulled out, sending CO_2 into the fume hood. The line was then flushed for 10 sec and the gas tank was turned off. The same procedure was used for introducing H_2 gas. Tubes were then pressurized with a gas mixture of different ratios of H_2/CO_2.

4.4.4 High pressure syringe studies

High pressure equipment were designed according to Takai and co-workers (2008) and manufactured by the University of Free State's (Instrumentation Department). Modifications were included to improve safety (pin indicated in the photo) and control

features to sustain effective constant pressure in vessels as shown in figure 4.6 A-B. Figure 4.6 represents the apparatus used for the completion of this work. A batch fluid cultivation system under high pressure was designed by using a combination of a glass syringe (Hamilton), a stainless steel needle and a butyl rubber stopper. All of the parts used, were repeatedly soaked and rinsed with 70% (v/v) EtOH and distilled water to minimize contamination during the experiments. The syringe incubator canisters were pressurized and the vessels were then incubated at 34˚C for *E. limosum* and 55˚C for the deep mine micro-organisms, in a temperature-controlled oven. The experiments were carried out in the syringe incubator canisters as described by Takai and co-workers (2008). These incubators are short term, and easy to do batch cultures in a gas tight high pressure vessel that makes use of a hydrostatic pump (Figure 4.6 D) to add pressure.

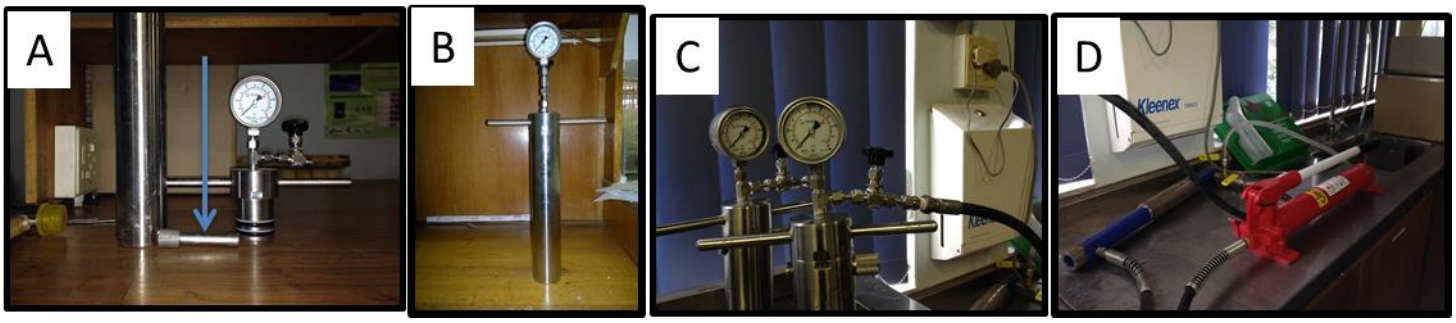

Fig.4.6. Apparatus used for the high pressure experiments and designs are based on the publication by (Takai *et al*., 2008) with modifications for safety and control.

Experiments were carried out as follows for the four selected organisms: Pressure was increased to determine maximum growth, while keeping the gas ratio constant at 20% CO_2 and 80% H_2. Once the ability to survive pressure was established, the CO_2 concentration was increased gradually until 100% CO_2, as described in section 4.4.2, while keeping pressure constant at 70 and 80 bar. Experiments were carried out at

70 and 80 bar because from 73 bar and above CO_2 is in its supercritical state at temperatures above 31˚C. In this state CO_2 then acts as a liquid-like density and a gas-like viscosity that can dissolve more freely in water and react with cations to form stable mineral compounds, such as hydrocarbons and methane. Each incubator is considered a sealed unit. This setup was done with 8 syringes and each harvested at the same time interval for analysis. This was done in duplicate. Two mL of liquid medium and cell inoculum, and various CO_2 and H_2 concentrations described in section 4.4.2 were added into the syringe. The serum vial, containing the gas mixtures at 2 bar, was equilibrated from the syringe by piston movement shown in figure 4.7 A. The cultivation syringes were then placed into the pressure canisters which were then compressed with a hydrostatic pump from 10 to 100 bar. Figure 4.7 B represents two canisters pressurized to 70 bar (non-supercritical conditions) and 80 bar (supercritical condition), and incubated at 34-55˚C for 48 hours. Samples were incubated in SC-CO_2 and non-SC-CO_2 conditions for approximately 48 hours. Growth was measured at an OD_{600} using the spectrophotometer (Spectronic® GENESYS 5). Experiments such as evaluation of survival using live/dead stain as described in section 3.6.4, pH and ATP analysis according to the manufacturer's instructions (CellTiter-Glo® Luminescent Cell Viability Assay, Promega) and exploratory HPLC analysis for acetate or formate production were conducted using the extracted culture from the syringes (Takai *et al*., 2008).

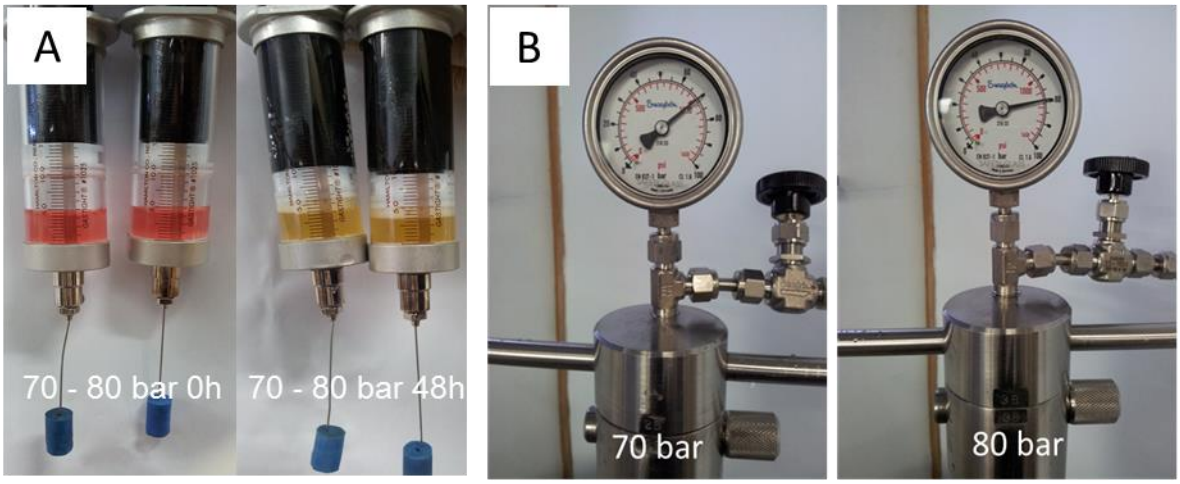

Fig.4.7. Hamilton syringes with 2 mL medium and 2 mL inoculum at 0 hours and 48 hours (A). Canisters are pressurized at 70 and 80 bar (B).

4.4.5 High performance liquid chromatography (HPLC) analysis for metabolic product detection

HPLC was performed with all of the selected micro-organisms at 20% CO_2 and 80% H_2 at 60 to 80 bar, and 100% CO_2 at 70 and 80 bar (in SC-CO_2 and non-SC-CO_2 conditions) from the high pressure syringe studies in section 4.4.4. By using 1 mL of culture that was centrifuged at 10 000 *x g* (Eppendorf, Centrifuge 5424) for ten minutes, the supernatant was collected and placed into HPLC tubes for analysis to explore formate and acetate production, which are products found in the reductive acetyl Co-enzyme A cycle. A Shimadzu Prominence chromatographic system was used with spectrophotometric detection at 202 nm. Separation was achieved with a Bio-Rad Aminex HPX 87H column, 300 mm x 7.8 mm and sulphuric acid, 5 mM, as eluent at a flow rate of 0.6 ml/min. The injection volume was 15 µL. The instrument was controlled and data collected with Shimadzu LabSolution software.

4.4.6 Gas chromatography (GC) analysis of CO_2 consumption quantification

A Tracera analytical instrument (Shimadzu) only arrived after release in October 2013. GC analysis was therefore, carried out on a Shimadzu 2010 chromatography fitted with a barrier discharge ionization detector. The analytical column was a Restek ShinCarbon ST AT/ 100 dimensions 2 m by 0.55 mm. Gas (headspace) of 250 µL from the Balch tube was injected with a gas tight syringe. Injection port temperature was 80˚C and the split ratio 50:1. Initial oven temperature was 40˚C held for two minutes then increased to 20˚C per minute to 150˚C. The carrier gas was Helium at 6.27 mL per minute and the detector temperature was set at 280˚C. Gas compositional analysis was done for *E. limosum* and *T. scotoductus* SA-01 to observe depletion of CO_2 in minimal media with and without glucose. Minimal medium was prepared as the following: 0.2 g/L KH_2PO_4, 0.25 g/L NH_4Cl, 1 g/L NaCl, 0.4 g/L $MgCl_2$, 0.5 g/L KCl, 0.1 g/L $CaCl_2$, 0.2 g/L MOPS, 0.1 g/L yeast extract, 1 mL/L vitamins, 1 mL/L trace elements at pH 7 with addition of 0.0003% (v/v) of resazurin and 1 g/L of glucose (So & Young, 1999). Medium preparation into Balch tubes are described in section 4.4.1. Gas was extracted with a 500 µL gas tight syringe from the Balch tubes and injected into the GC port for analysis.

4.5 Results and discussion

4.5.1 Low pressure studies

Low pressure growth studies were performed according to section 4.4.1 for the three

deep mine micro-organisms at 2 bar with 20% CO_2 and 80% H_2 for 48 hours and *E. limosum* 56 hours in Balch tubes. *E. limosum* was selected because of its ability to grow at 2 bar (Genthner *et al*., 1981); no reports or the ability to endure pressure have been described in the other micro-organisms. The growth of *E. limosum* showed a longer lag phase under these conditions but growth confirmed that this organism grows at 2 bar at 20% CO_2 and 80% H_2 to an OD valve of at least 1 with a specific growth rate of 0.13 per hour. *E. limosum* reached late exponential phase in approximately 56 hours. Cells were stained using the Live/dead® BacLight TM Bacterial Viability Kit, as described in section 3.6.4, to confirm the viability of the micro-organisms at 2 bar with 20% CO_2 and 80% H_2 (Figure 4.8). The Live/dead stain was performed to determine if *E. limosum* was still viable when late exponential phase was reached. The stain confirmed comprehensively that the majority of cells were alive. However, from figure 4.9, it can be seen that the three deep mine micro-organisms did not display significant growth, as expected from section 3.9.4 where no production of biomass was seen in anaerobic growth studies (*T. scotoductus* SA-01 [Blue line], *Geobacillus* sp. GE-7 [Green line] and *Geobacillus* sp. A12 [Red line]). Live/dead stain was performed as described in section 3.6.4, (Figure 4.9 A-C) and surprisingly the three deep mine micro-organisms *T. scotoductus* SA-01 (A), *Geobacillus* sp. A12 (B) and *Geobacillus* sp. GE-7 (C) were still alive at even after exposure to 2 bar with 20% CO_2 and 80% H_2 for 25 hours. However, there were more dead cells at 2 bar with 20% CO_2 and 80% H_2 growth studies in comparison to aerobic growth studies for these three micro-organisms in chapter 3.

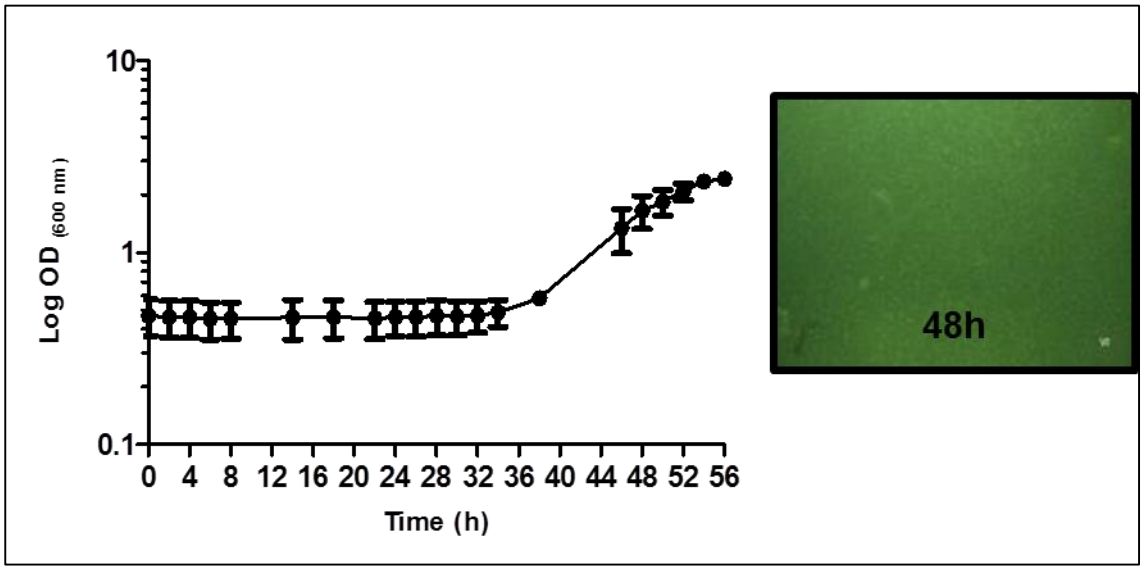

Fig.4.8. Growth curve for *E. limosum* at 2 bar with 20% CO_2 and 80% H_2 where optical density was monitored over time. Scale bars was set at 2 µm

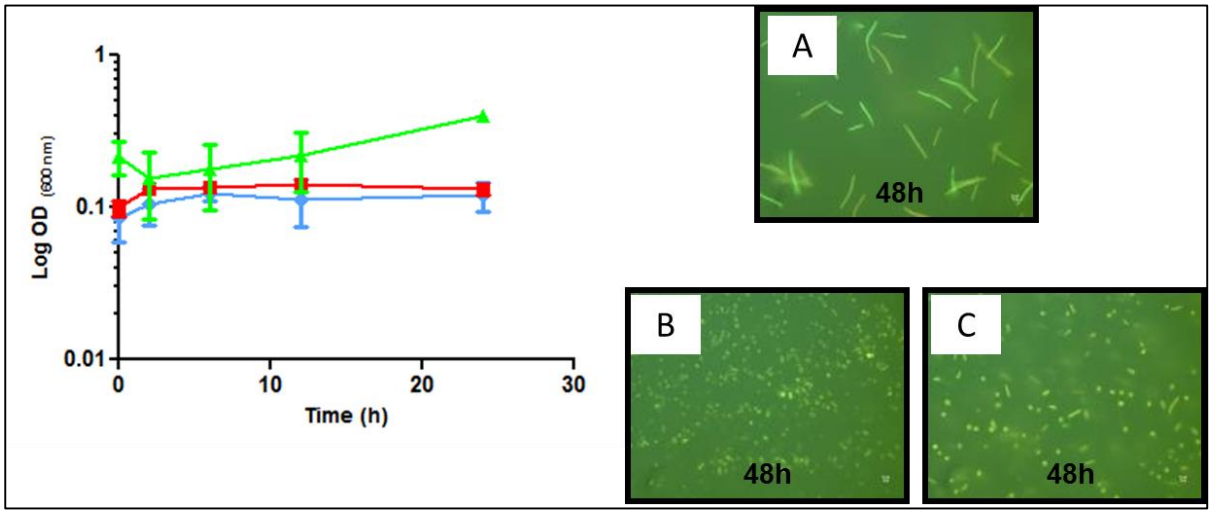

Fig.4.9. Growth curves for the three selected mine micro-organisms at 2 bar with 20% CO_2 and 80% H_2 where optical density was monitored over time for 25 hours. Scale bars were set at 2 µm. *T. scotoductus* SA-01 (A) (Blue line), *Geobacillus* sp. GE-7 (B) (Green line) and *Geobacillus* sp. A12 (C) (Red line).

4.5.2 High pressure syringe studies

High pressure growth studies were performed as described in section 4.4.4. *E. limosum*, known to grow at 2 bar (Genthner *et al*., 1981) was introduced to pressures

associated with CCS conditions from 10 to 100 bar, increasing in 10 bar increments, under 20% CO_2 and 80% H_2 to evaluate their survival. Cells were stained using the Live/dead® BacLight TM Bacterial Viability Kit as described in section 3.6.4, to confirm the viability of the micro-organisms at selected parameters (Figure 4.10). Results indicated that the cells were still alive after 48 hours even at 100 bar with 20% CO_2 and 80% H_2. Research in the past only indicated that this organism grows up to 2 bar with 20% CO_2 and 80% H_2. However, once the ability to survive increasing pressure was established, the CO_2 concentration was increased gradually until 100% CO_2, as described in section 4.4.2, while keeping pressure constant at 70 and 80 bar (SC-CO_2 and non- SC-CO_2 conditions). Results from the live/dead stain indicated that the cells were still alive even with 50% CO_2, 80% CO_2 and 100% CO_2 after 48 hours shown in figures 4.11-4.13. According to literature, *E. limosum* is able to use H_2 and CO_2 to synthesize acetate, and by using the reductive acetyl-CoA pathway to form formate (Lelait & Grivet, 1996; Leclerc *et al*., 1997). The pH values at 0 hours were approximately around 6.15 to 6.24 and at 48 hours around 4.88 to 5.25 for all the different CO_2 concentrations. This indicated that the medium is more acidic at 48 hours which means acids such as formate and acetate may be formed. Metabolic activity (ATP) was monitored during these tests and the results for *E. limosum* indicate that this organism remained metabolically active. Most surprisingly this organism was able to remain viable and metabolically active even at 100 bar and 100% CO_2. This has never been reported in literature before.

20% CO_2 and 80 % H_2

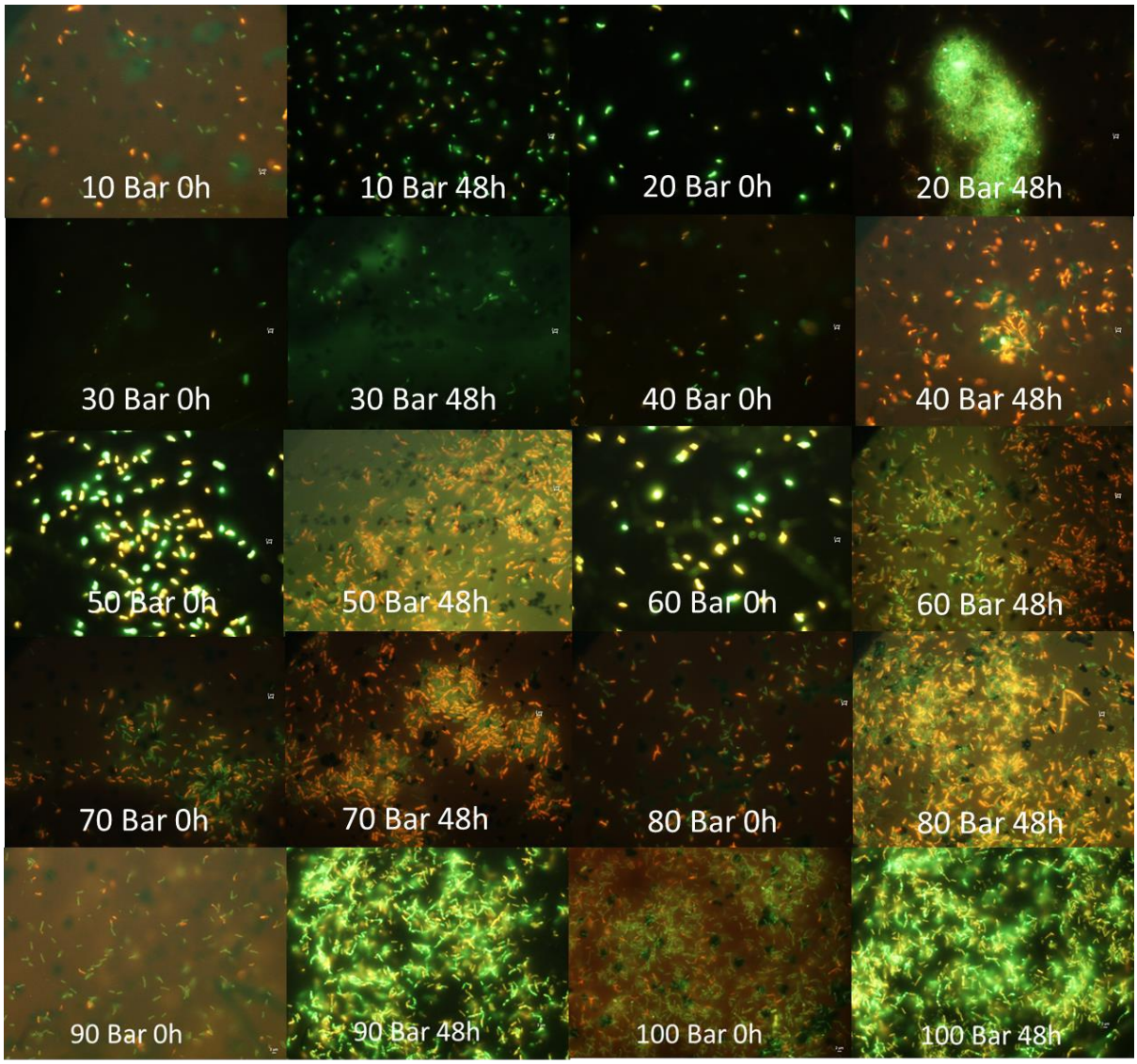

Fig.4.10. Live/dead stain was performed to determine if *E. limosum* was still viable at 20% CO_2 and 80% H_2 from 10 to 100 bar. Scale bars were set at 2 µm.

50% CO_2 and 50 % H_2

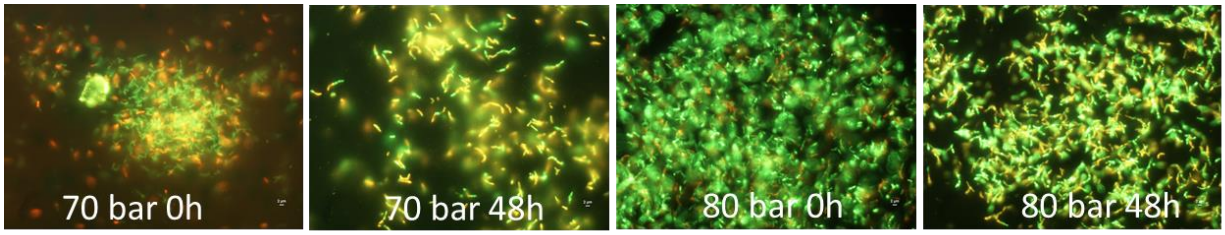

Fig.4.11. Live/dead stain was performed to determine if *E. limosum* was still viable at 50% CO_2 and 50% H_2 at 70 and 80 bar. Scale bars were set at 2 µm.

80% CO_2 and 20 % H_2

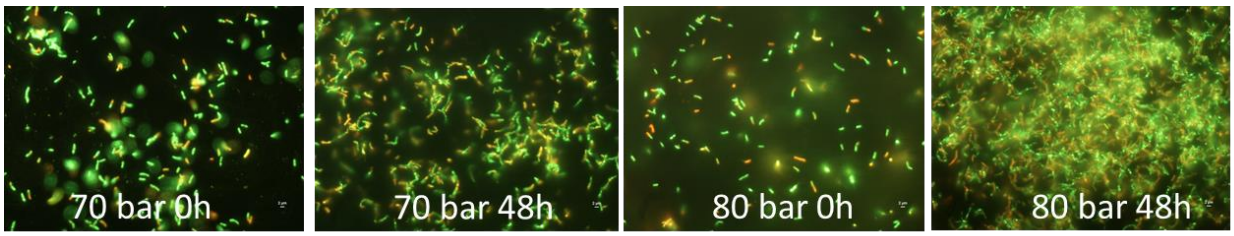

Fig.4.12. Live/dead stain was performed to determine if *E. limosum* was still viable at 80% CO_2 and 20% H_2 at 70 and 80 bar. Scale bars were set at 2 µm.

100% CO_2

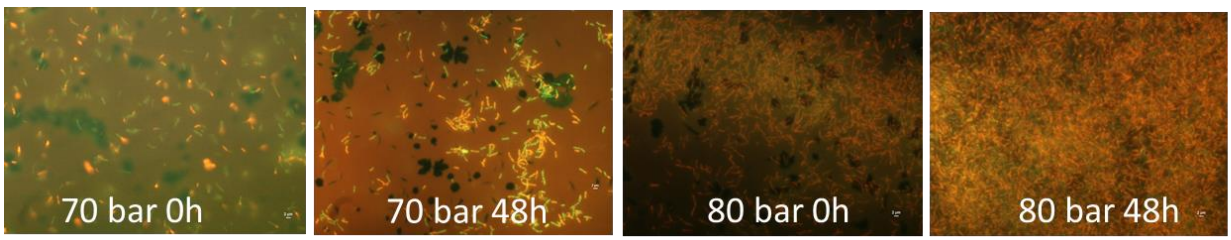

Fig.4.13. Live/dead stain was performed to determine if *E. limosum* was still viable at 100% CO_2 at 70 and 80 bar. Scale bars were set at 2 µm.

Since *T. scotoductus* SA-01 showed that it can remain viable at 2 bar with 20% CO_2 and 80% H_2 (Section 4.5.1), it was subjected to increasing pressures in 20 bar increments, with 20% CO_2 and 80% H_2, to evaluate survival. Cells were stained using the Live/dead® BacLight TM Bacterial Viability Kit as described in section 3.6.4, to

confirm the viability of the micro-organisms at different parameters (Figure 4.14). Results indicated that the cells were still alive after 48 hours even at 100 bar with 20% CO_2 and 80% H_2. Research in the past never indicated that this organism can grow at any pressure parameters or at any CO_2 concentrations. However, once the ability to survive increasing pressures was established, the CO_2 concentration was increased gradually until 100% CO_2, as described in section 4.4.2, while keeping pressure constant at 70 and 80 bar (SC-CO_2 and non-SC-CO_2 conditions). Results from the live/dead stain indicated that the cells were still alive even with 50% CO_2, 80% CO_2 and 100% CO_2 after 48 hours, shown in figures 4.15-4.17. Gounder and co-workers (2011), identified that *T. scotoductus* SA-01 retains the reductive tricarboxylic acid cycle (TCA) and the genome retrieved using Metacyc database collection (Altman *et al*., 2013) also indicate that *T. scotoductus* SA-01 is capable of CO_2 fixation, which means acids such as formate and acetate may be formed. The pH values remained approximately around 6.62 to 6.91 at 0 hours and at 48 hours approximately 6.6 to 7.03 for all the different CO_2 concentrations. Metabolic activity (ATP) was monitored during these tests and results for *T. scotoductus* SA-01 indicated that this organism remained metabolic active but at very low rates. These results were as expected due to the anaerobic growth study performed in section 3.9.4 and the 2 bar with 20% CO_2 and 80% H_2 in section 4.5.1 where there was no indication of biomass production after a period of time but the cells remained viable. Most surprisingly, this organism was able to remain viable and metabolically active even at 100 bar and 100% CO_2. This has never been reported before in literature. This indicates that *T. scotoductus* SA-01, found in the subsurface, could interact with pressures and CO_2 concentrations associated with CCS conditions which may contribute towards carbon cycling.

20% CO_2 and 80 % H_2

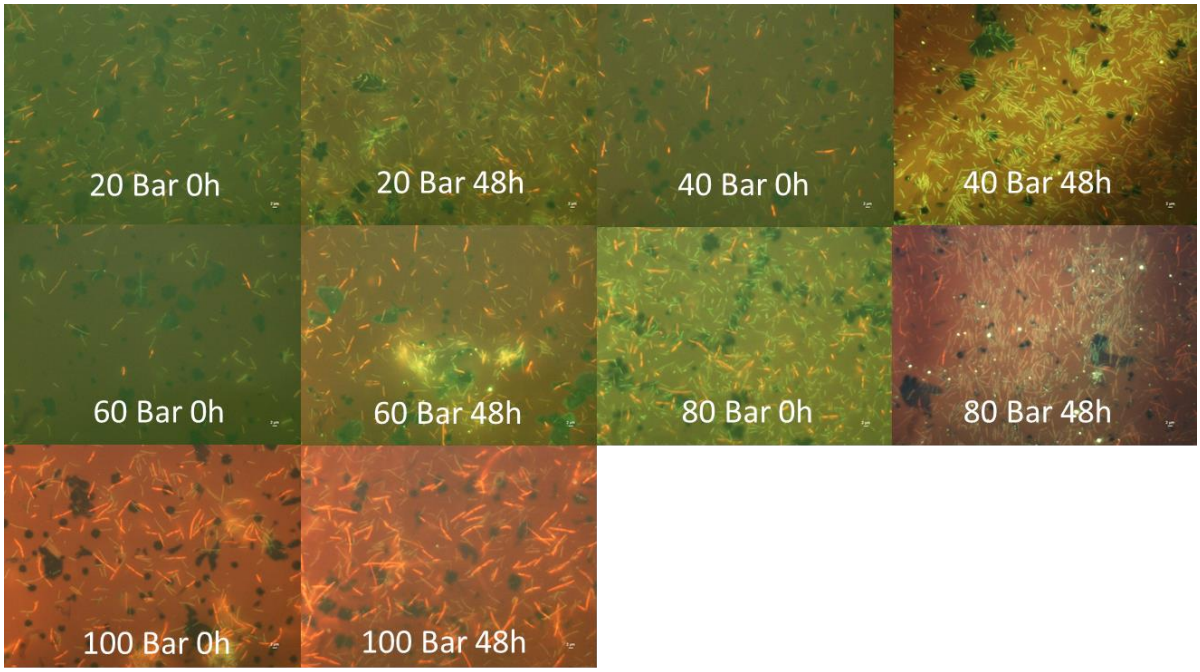

Fig.4.14. Live/dead stain was performed to determine if *T. scotoductus* SA-01 was still viable at 20% CO_2 and 80% H_2 from 20 to 100 bar. Scale bars were set at 2 µm.

50% CO_2 and 50 % H_2

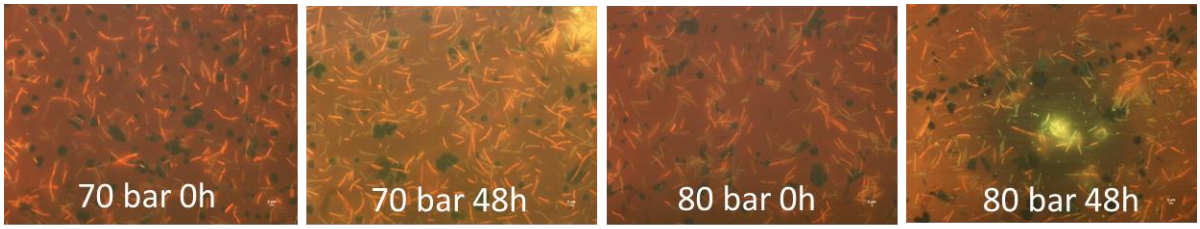

Fig.4.15. Live/dead stain was performed to determine if *T. scotoductus* SA-01 was still viable at 50% CO_2 and 50% H_2 at 70 and 80 bar. Scale bars were set at 2 µm.

80% CO_2 and 20 % H_2

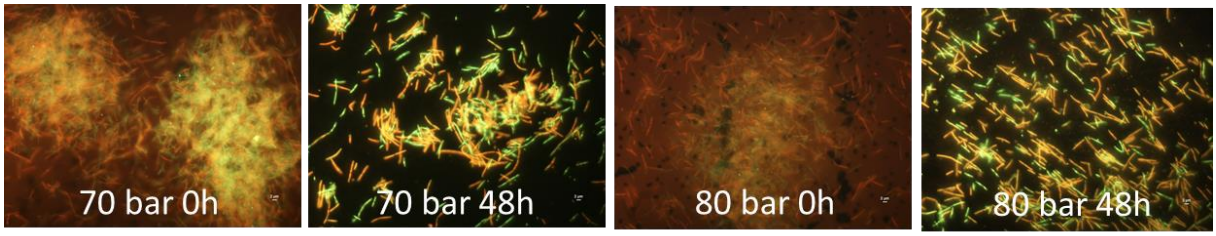

Fig.4.16. Live/dead stain was performed to determine if *T. scotoductus* SA-01 was still viable at 80% CO_2 and 20% H_2 at 70 and 80 bar. Scale bars were set at 2 µm.

100% CO_2

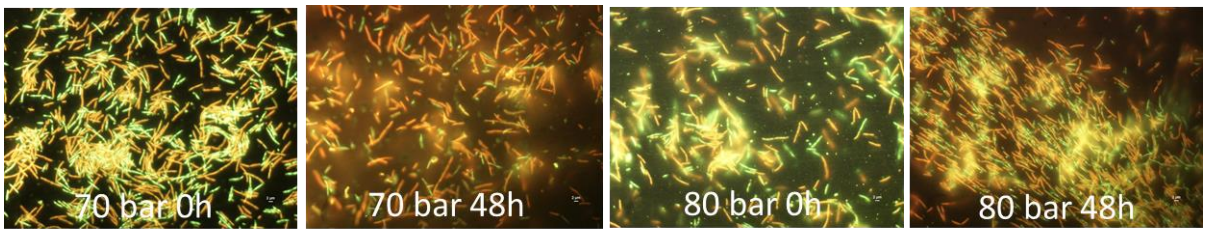

Fig.4.17. Live/dead stain was performed to determine if *T. scotoductus* SA-01 was still viable at 100% CO_2 at 70 and 80 bar. Scale bars were set at 2 µm.

Since *Geobacillus* sp. GE-7 and *Geobacillus* sp. A12 showed that it can remain viable at 2 bar with 20% CO_2 and 80% H_2 (Section 4.5.1), they were subjected to increasing pressures in 20 bar increments with 20% CO_2 and 80% H_2 to evaluate their survival. Cells were stained using the Live/dead® BacLight TM Bacterial Viability Kit as described in section 3.6.4, to confirm the viability of the micro-organisms at different parameters (Figure 4.18 A-B). The stain confirmed that the cells were dead at 48 hours for both *Geobacillus* sp. A12 (A) and *Geobacillus* sp. GE-7 (B) from 20 to 80 bar at 20% CO_2 and 80% H_2. However, since these micro-organisms could not survive increasing pressure at CO_2 concentration of 20% CO_2 and 80% H_2, a set of no gasses was included and only pressures as described in section 4.4.4 from 20 to 80 bar was introduced. The control incubation where no CO_2

was added and pressure was introduced using the hydrolytic pump showed that the cells were dead after 48 hours for both *Geobacillus sp.* A12 (A) and *Geobacillus* sp. GE-7 (B) (Figure 4.19 A-B). The pH values for the media of *Geobacillus* sp. GE-7 remained approximately around 7.2 to 7.6 at 0 hours and at 48 hours approximately 6.95 to 7.3 for 20% CO_2 and 80% H_2, as well as when no gasses were included, thus no chemical parameters were introduced that affected survival. The pH values for *Geobacillus* sp. A12 remained approximately around 6.85 to 6.98 at 0 hours and at 48 hours approximately 6.5 to 6.4 for 20% CO_2 and 80% H_2 and when no gas mixture was included. Metabolic activity (ATP) was monitored during these tests and results for both *Geobacillus* sp. GE-7 *and Geobacillus* sp. A12 indicate that these organisms were not metabolically active at 48 hours from 20 bar to 80 bar at 20% CO_2 and 80% H_2, as well as when no gasses was included. These results were as expected due to the anaerobic growth study performed in section 3.9.4 and the 2 bar with 20% CO_2 and 80% H_2 in section 4.7.1 where there was no indication of biomass production after a period of time. However, this indicates that both *Geobacillus* sp. GE-7 and *Geobacillus* sp. A12 are very sensitive to pressure and that these two micro-organisms cannot survive under increasing pressure or/and when 20% CO_2 and 80% H_2 was introduced. According to literature, *Geobacillus stearothermophilus* was shown to remain viable at 30 bar for a short period of time (Santillan *et al*., 2012). Research in the past has never introduced these two micro-organisms to any pressure parameters or at any CO_2 concentrations.

20% CO_2 and 80 % H_2

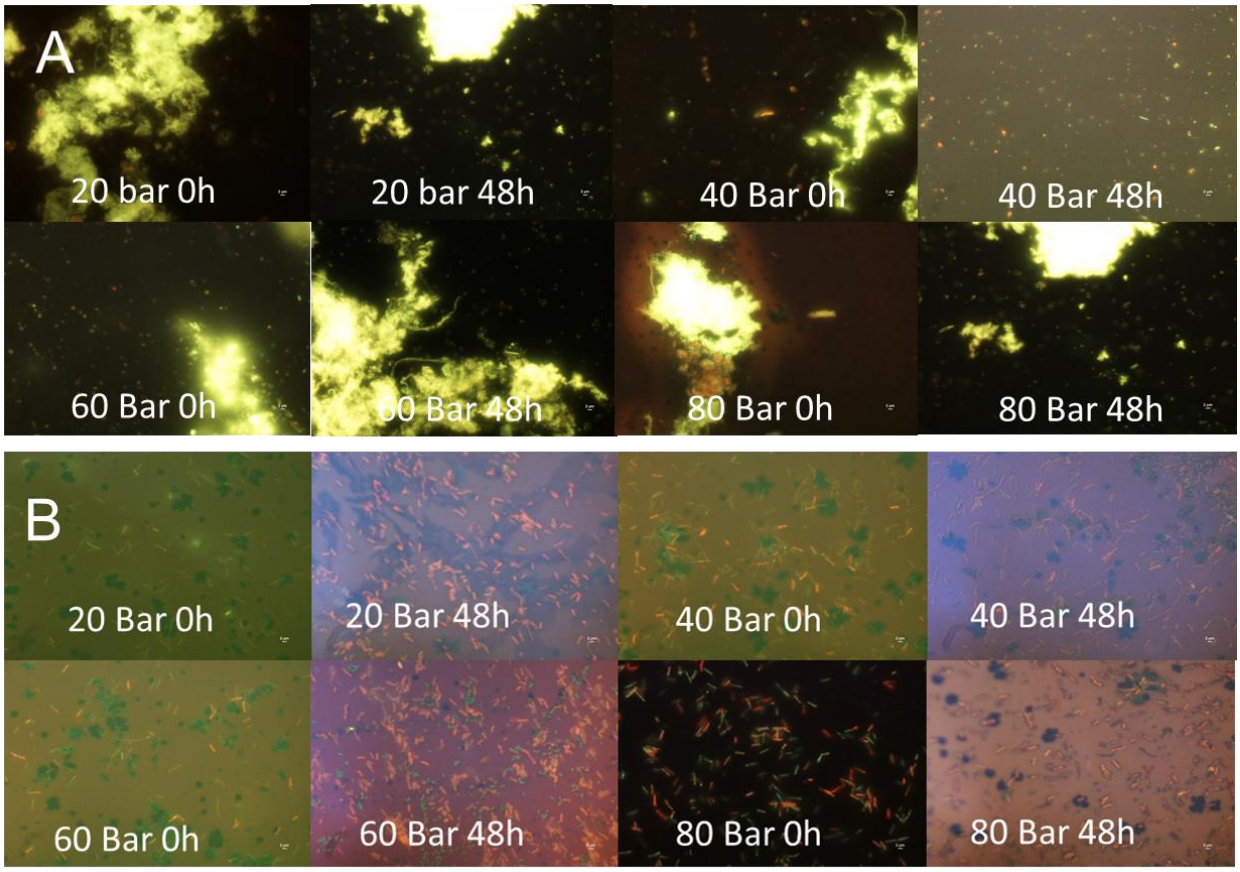

Fig.4.18. Live/dead stain was performed to determine if *Geobacillus* sp. A12 (A) and *Geobacillus* sp. GE-7 (B) were still viable at 20% CO_2 and 80% H_2 from 20 to 80 bar. Scale bars were set at 2 µm.

Growth with pressure and no gas

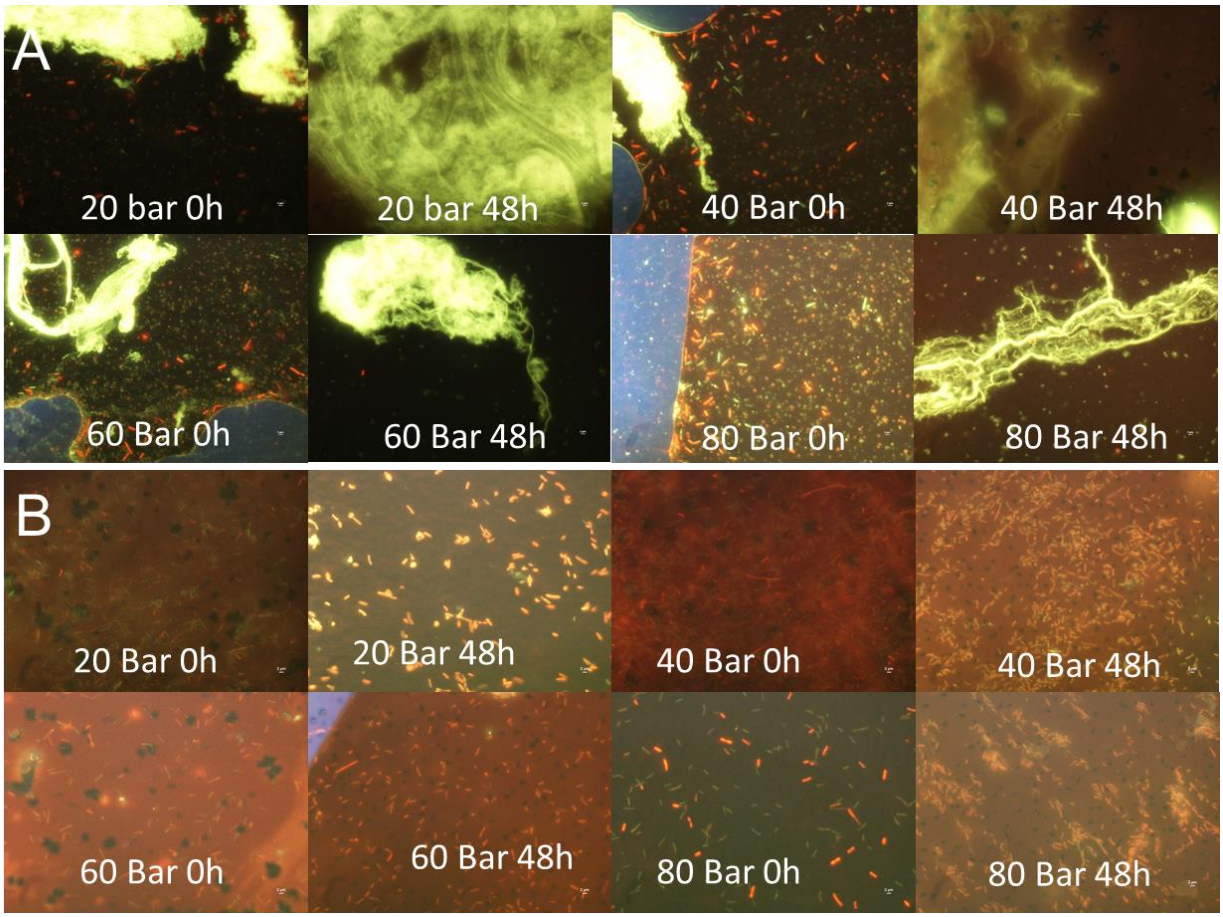

Fig.4.19. Live/dead stain was performed to determine if *Geobacillus* sp. A12 (A) and *Geobacillus* sp. GE-7 (B) were still viable when no gasses are included from 20 to 80 bar. Scale bars were set at 2 µm.

The stains on the four micro-organisms suggest that cell survival at increasing pressure may be dose dependent on CO_2. The amount of dead cells increased with increasing amounts of CO_2, suggesting a critical concentration that affects the cell's survival ability. Therefore, cell death may be due to the dissolved CO_2 in solution and not just the pressure of CO_2 exerted on the organisms (Santillan *et al*., 2013). Microbial tolerances towards high CO_2 pressures vary from organism to organism due to environmental, biochemical and structural characteristics (Hong & Pyun, 1999; Spilimbergo & Bertucco 2003; Watanabe *et al*., 2003). As described in section 4.2, Popper and Knorr (1990) stated that the morphology and sizes of the micro-

organisms were factors in resistance towards pressurized CO_2. Gram-positive bacteria seems to have more resistance towards pressurized CO_2, compared to Gram-negative bacteria, because of their thick layer of peptidoglycan that slows down CO_2 penetration into the cell. However, results indicated that the Gram-positives *Geobacillus* sp. GE-7 and *Geobacillus* sp. A12 cannot survive under increasing pressures for 48 hours even if no gas mixture was added. This is not seen for the other Gram-positive microorganism, *E. limosum*.

Oulé and co-workers (2010) described that the microbicidal activity of CO_2 is based on pressure, temperature and the type of micro-organisms, and the resistance of the bacteria towards SC-CO_2 depends on their morphology, structure and physiology. Thus, the difference between the Gram-positive bacteria may be due to their different tolerance towards pressure and their abilities to deal with carbon dioxide fixation and should be coupled to their metabolic capabilities.

If carbon fixation was active one would expect acetate and formate production (Reductive acetyl Coenzyme A pathway). HPLC analysis was carried out according to section 4.4.5. Figure 4.20 displays the HPLC analysis for acetate and formate formation. Unfortunately the results indicate that no significant amount of organic acids, such as acetate and formate are present in the medium at 20% CO_2 and 80% H_2 (60 to 80 bar), and 100% CO_2 (70 and 80 bar). This might be due to detection limits and one should consider the low concentration of substrate available for the micro-organisms and turnover.

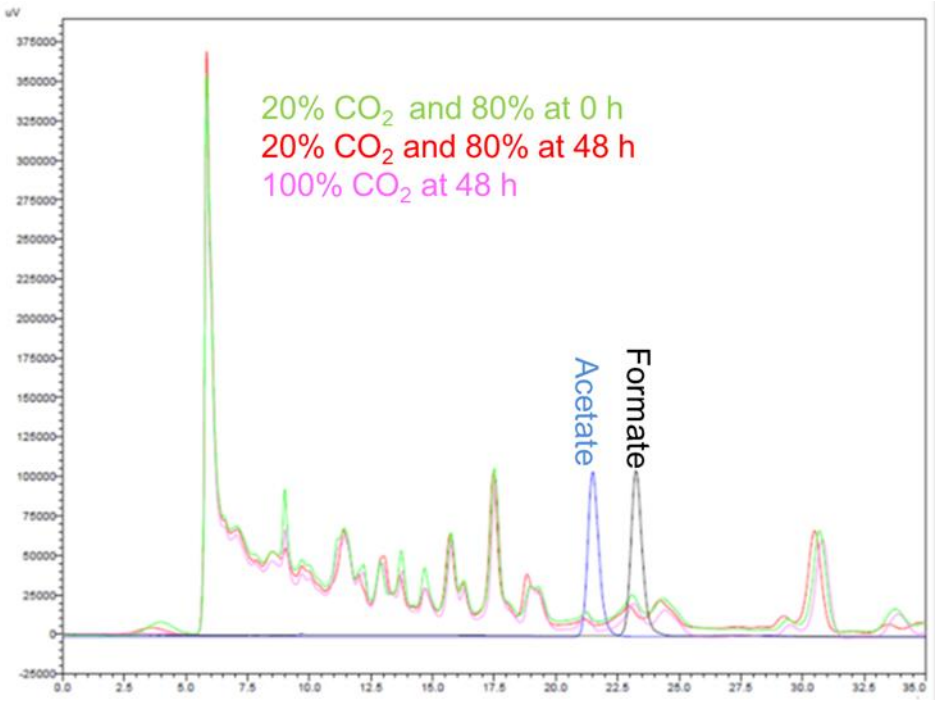

Fig.4.20. HPLC analysis for *E. limosum* at 20% CO_2 and 80% H_2 at 0 hours in green, 48 hours in red and 100% CO_2 in pink. There were no indications of formation of acetate or formate formation.

4.5.3 Low pressure studies with minimal medium

E. limosum and *T. scotoductus* SA-01 were shown to remain viable at 100% CO_2 at supercritical and non-supercritical conditions (Section 4.5.2). Thus, the two micro-organisms were inoculated into minimal media, with and without glucose to evaluate CO_2 consumption via carbon fixation pathways by GC analysis. Since it was shown that the micro-organisms even after 48 hours have ATP available (energy for fixation process?) as well as the reactive pathways in their genomes. The genomes of the two micro-organisms were retrieved and compared using Metacyc database collection (Altman *et al*., 2013), which is a database that provides all the metabolic pathways available for the organisms. Both of the organisms can fix carbon dioxide using the reductive citric acid cycle (rTCA), the reductive acetyl-CoA pathway (rAcCoA) and for *E. limosum* the Calvin cycle is also available to fix more CO_2.

Metabolic activity (ATP) was monitored during these tests. According to literature *E. limosum* can utilize a wide range of carbon sources such as multi-carbon substrates including hexoses, pentose sugars, lactate, dihydroxyacetone, and one-carbon compounds such as methanol, formate, carbon monoxide, H_2/CO_2 (Bloas *et al*., 1993; Chang *et al*., 2001). This might be an explanation why *E. limosum* grows better in minimal media with glucose, where more ATP production can be achieved. The ATP measurements after the 6 days of incubation in media supplemented with glucose (14842.90 RLU) *vs*. no additional supplement (912.07 RLU), clearly indicates additional ATP availability. Since all fixation pathways are ATP dependant the correlation of final CO_2 consumption shows better utilization when higher ATP amounts were present. *T. scotoductus* SA-01 also has access CO_2 fixation pathways, however, *T. scotoductus* SA-01 showed lower rates of ATP production in minimal medium especially with glucose.

However, results obtained from the GC analysis were inconsistent due to possible leakage of the Balch tubes stoppers when pressurized and sampled. Thus, makes the starting and final analysis difficult to standardize for quantitative analysis. Table 4.2 displays the N_2 standard and N_2 (Ar) internal standard, which were supposed to remain constant for both 0 hours and 48 hours to be able to calculate the CO_2 consumption by the organisms. Due to variation of internal standards, final CO_2 consumptions rates cannot be calculated using equation 1.

Table 4.2: Internal standard for GC analysis.

Sample	N_2 0 Hours	N_2 (Ar) 0 Hours
MM Control	2148332	3241022
MM Control	2507730	1777175
MM SA-01	234706	539593
MM SA-01	2331757	1314983
MM E.lim	3269655	3186438
MM E.lim	902491	1677512
MM+ G	383041	521006
MM+ G	4090799	3021044
MM+ G SA-01	1429560	703022
MM+ G E.lim	1618370	1232953
MM+ G E.lim	2101290	1390672
	N_2 48 Hours	N_2 (Ar) 48 Hours
MM Control	5950041	3317604
MM Control	5161320	4339274
MM SA-01	4181531	3477456
MM SA-01	4551968	4076125
MM E.lim	3963373	3781817
MM E.lim	4597016	5020229
MM+ G	8707046	3870263
MM+ G	4649188	3416641
MM+ G SA-01	4757149	4198088
MM+ G E.lim	4211147	4191901
MM+ G E.lim	4292078	4368587

Cells were stained using the Live/dead® BacLight TM Bacterial Viability Kits as described in section 3.6.4 to confirm the viability of the micro-organisms after six days represented in figure 4.20 and 4.21. The stains confirmed that some of the cells were alive at 48 hours at 2 bar with 100% CO_2 in minimal media with and without glucose (*E. limosum* [A] and *T. scotoductus* SA-01 [B])

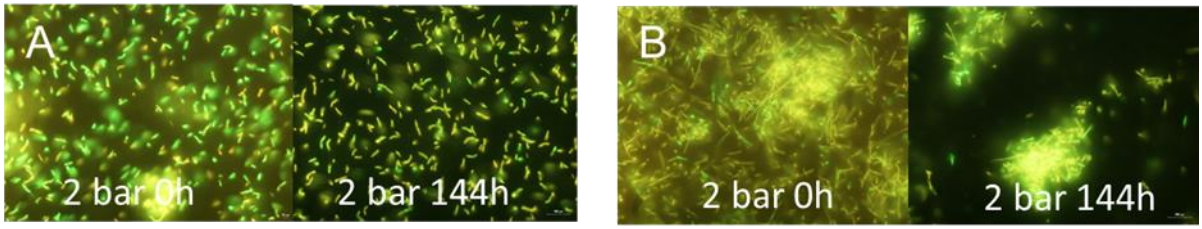

Fig.4.21. Live/dead stain performed to determine if *E. limosum* (A) and *T. scotoductus* SA-01 (B) was still viable at 100% CO_2 at 2 bar with minimal media. Scale bars were set at 2 µm.

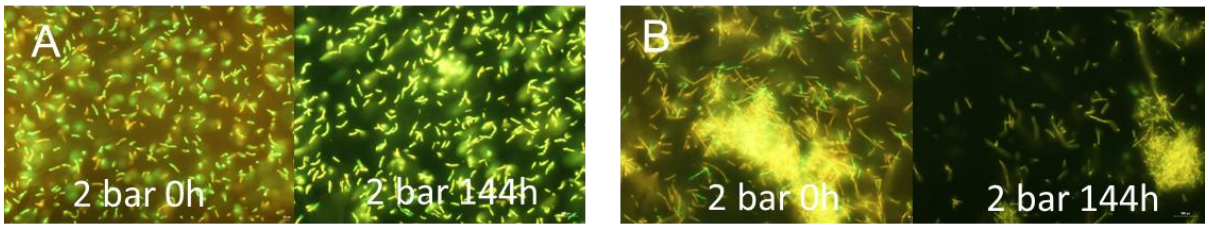

Fig.4.22. Live/dead stain performed to determine if *E. limosum* (A) and *T. scotoductus* SA-01 (B) was still viable at 100% CO_2 at 2 bar with minimal, containing glucose, media. Scale bars were set at 2 µm.

4.6 Conclusions

The Gram-negative, *T. scotoductus* SA-01, and the Gram-positives, *Geobacillus* sp. GE-7, *Geobacillus* sp. A12 and *E. limosum*, were all tested for their ability to survive under low and high pressures as well as exposure towards CO_2 at different concentrations. Pressure was applied to all four micro-organisms from 2 to 100 bar. Live/dead stain and analytical tests were done to identify and observe if these micro-organisms are able to withstand pressure and produce expected compounds such as acetate and formate. The four micro-organisms showed survival at 2 bar with 20% CO_2 and 80% H_2. *E. limosum* has been known to grow at 2 bar as described in literature. However, most surprisingly *T. scotoductus* SA-01, *Geobacillus* sp. GE-7 and *Geobacillus* sp. A12 were able to remain viable and metabolically active at 2 bar. This has never been reported in literature before. Since the four micro-organisms showed survival at 2 bar with 20% CO_2 and 80% H_2, the pressure and CO_2 concentration was increased. The results obtained for *E. limosum* and *T. scotoductus* SA-01, confirmed that they were able to remain viable and metabolically active at even 100 bar with 100% CO_2, whereas *Geobacillus* sp. GE-7 and *Geobacillus* sp. A12 could not survive pressures above 2 bar. GC analysis for both *E. limosum* and *T. scotoductus* SA-01 indicated that CO_2 was depleted over a period of time. This indicates that *T. scotoductus* SA-01 found in the subsurface and *E. limosum* could withstand pressures and CO_2 concentrations associated with CCS conditions which could contribute towards carbon cycling and should therefore be considered in the future in test simulations.

4.7 References

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CHAPTER 5

CHAPTER 5

GENERAL CONCLUSIONS

5.1 Conclusions

The economy of South Africa is mainly driven by the usage of coal which contributes to global warming, causing climate change around the world. In 2009, the Department of Environmental Affairs announced that CO_2 emissions in South Africa will increase until 2020-2025 and followed by a decrease after 2030-2035 (CO_2 capture, transport and storage, 2009; Cloete, 2010). The aim of carbon sequestration is to limit CO_2 emissions into the atmosphere by storing the CO_2 in oceans or geological sites. Storing CO_2 in the subsurface could directly impact the deep subsurface microbial ecosystems and the biogeochemical processes. The understanding of carbon capture and storage in the deep biosphere and the behaviour of CO_2 are limited. However, storing CO_2 at depths of 1 000 m and temperatures above 31˚C will cause the CO_2 to be in its supercritical state (Holloway, 2007).

The main aim of this project was to use known, deep subsurface micro-organisms and a positive control that was known to grow under pressure and utilize CO_2, and to observe if they were able to withstand carbon sequestration conditions. Experiments were done to confirm if these selected subsurface micro-organisms were able to stay alive and remain metabolically active under pressure and supercritical CO_2 conditions. Careful considerations should be given to *in situ* subsurface cycling

during terrestrial Carbon capture and storage

Micro-organisms that live in extreme environments, such as high temperatures and anaerobic or acidic conditions, generally utilize different CO_2 fixation pathways (Johnston *et al*.,1999; Kharaka *et al*., 2009; Velea *et al*., 2009; Graber, 2011; West *et al*., 2011). Micro-organisms isolated from the deep subsurface that were included in this study are, *Thermus scotoductus* SA-01 isolated by Kieft and co-workers (1999), *Geobacillus thermoleovorans* GE-7 isolated by DeFlaun and co-workers (2007) and *Geobacillus thermoparaffinivorans* A12 isolated by Jugdave (2011). In addition, a microorganism that is known to grow under 2 bar pressure and utilize CO_2, *Eubacterium limosum* (Genthner *et al*., 1981) was also selected as representative to illustrate CO_2 utilization under increasing pressure. The identities of the selected micro-organisms were verified through 16S rRNA gene amplification, cloning and sequencing. Available genome sequences of these micro-organisms were compared and a few metabolic pathways were identified in their respective genomes. The CO_2 fixation pathways of interest were the Calvin cycle, the reductive acetyl Co-enzyme A cycle and the reductive citric acid cycle. All the micro-organisms retain the three cycles mentioned, except for *Thermus* SA-01 where the Calvin cycle is not present.

These organisms were identified and explored to understand if they were capable of survival under increasing pressure and remain active at different CO_2 concentrations. Organisms were grown in a closed system using high pressure syringe incubators for 48 hours. A hydrostatic pump was used to add pressures ranging from 20 bar to 100 bar. Cultures were then stained using the BacLight live/dead staining kit and tested

for metabolic activity such as ATP, production of acetate, formate and CO_2 utilization.

The selected micro-organisms were isolated from different areas and depths within the subsurface. Thus, they likely have different tolerances towards pressure and adaptation. *Geobacillus* sp. are found in the deep mine. Information regarding the metabolic pathways for *Geobacillus* sp. GE-7 and *Geobacillus* sp. A12 were retrieved from Metacyc database collection (Altman *et al*., 2013) indicating that they were capable to fix CO_2. Popper and Knorr (1990) stated that Gram-positive bacteria seems to have more resistance towards pressurized CO_2 when compared to Gram-negative bacteria because of its thick layer of peptidoglycan that slows down CO_2 penetration into the cell. According to literature, *Geobacillus stearothermophilus* can remain viable at 30 bar for a short period of time (Santillan *et al*., 2012). However, *Geobacillus* sp. GE-7 and *Geobacillus* sp. A12 could only sustain themselves under 2 bar pressure but did not remain viable with pressures higher than 20 bar. These results were not as expected since the metabolic pathway, as well as the environments where these micro-organisms were found, indicated high possibilities of adaption to CCS conditions. *E. limosum* can, according to available literature, remain viable under 2 bar pressure. Surprisingly this organism was able to remain viable and metabolically active at 100 bar pressure in the presence of 100% CO_2. Similar results were also obtained for *T. scotoductus* SA-01. Survival under these conditions has not been described for these two organisms. Information regarding the metabolic pathways for *T. scotoductus* SA-01 and *E. limosum*, retrieved from Metacyc database collection (Altman *et al*., 2013) also indicate that the CO_2 fixation pathways mentioned before, are available.

The aims of this study, specifically to identify if these micro-organisms were able to survive under increasing pressures and different CO_2 concentrations associated with terrestrial sequestration, were successfully completed. Results obtained indicate that the interactions between supercritical CO_2 and the individual organisms are still relatively unknown and should be considered when CCS test sites are injected.

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CHAPTER 6

CHAPTER 6

SUMMARY

South Africa‘s economy is primarily driven by the utilization of coal to provide electricity, which results in more fossil fuels to be burnt that contributes towards global warming. The average daily temperature is estimated to rise between 1.1 to 6.4˚C by 2100. Carbon sequestration is a technology that can limit CO_2 emission into the atmosphere by storing the CO_2 away in oceans or the terrestrial subsurface. South Africa is focusing on geological storage at depths of 1 000 m. Limited scientific knowledge is available on the direct impact when large amounts of supercritical CO_2 is injected into the subsurface. This includes the diversity of the deep subsurface microbial communities as well as their ecosystems and biogeochemical processes.

The main aim of this project was to use selected deep subsurface micro-organisms (*T. scotoductus*, *Geobacillus* sp. GE-7 and *Geobacillus* sp. A12) and an organism that was known to grow under pressure (*E. limosum*) and introduce them to CCS conditions using a high pressure syringe incubator system.

The identities of the selected micro-organisms were verified using molecular techniques, the genomes of these micro-organisms were retrieved and information regarding possible CO_2 fixation pathways was verified using the Metacyc database collection. The CO_2 fixation pathways of interest were the Calvin cycle, the reductive acetyl Co-enzyme A and the reductive citric acid cycles.

Surprisingly, *T. scotoductus* and *E. limosum* were able to remain viable and metabolically active even at 100 bar and 100% CO_2. This has never been previously reported in literature. However *Geobacillus* sp. GE-7 and *Geobacillus* sp. A12 could not remain viable when the pressure was increased from 2 bar to 20 bar or higher.

The outcomes of this study indicate that the interactions between supercritical CO_2 and the subsurface organisms should be considered as biogeochemical cycling. However, these interactions in the subsurface are still relatively unknown and the availability of interactive metabolic pathways indicate that the subsurface communities could survive and interact with this introduced substrate.

Key words: Carbon sequestration, Supercritical CO_2, Pressure, Metabolic pathways, Deep mine micro-organisms, *Eubacterium limosum*.

CHAPTER 6

OPSOMMING

Suid-Afrika se ekonomie word hoofsaaklik gedryf deur die gebruik van steenkool om elektrisiteit te voorsien, wat veroorsaak dat meer fossielbrandstof gebruik word wat bydra tot aardverwarming. Daar word voorspel dat die gemiddelde daaglikse temperatuur tussen 1.1 tot 6.4˚C sal verhoog voor 2100. Koolstofsekwestrasie is ‘n tegniek wat CO_2 vrystelling in die atmosfeer kan beperk deur die berging van CO_2 in oseane en onder die grond. Suid-Afrika fokus op die geologiese berging van CO_2 by dieptes van 1 000 m. Beperkte wetenskaplike kennis is beskikbaar oor die direkte impak van groot hoeveelhede superkritiese CO_2 wat diep in die grond geberg word. Dit sluit die mikrobiese gemeenskap asook die ekosisteme en biogeochemiese prosesse in.

Die doel van die projek was om geselekteerde diep-myn mikroörganismes (*T. scotoductus*, *Geobacillus* sp. GE-7 en *Geobacillus* sp. A12) asook *E. limosum*, ‘n mikroörganisme wat bekend is om onder hoë druk te groei, bekend te stel aan CCS toestande, deur gebruik te maak van ‘n hoë druk sisteem.

Geselekteerde mikroörganismes was deur middel van molekulêre tegnieke ge-identifiseer, die genome van hierdie organismes was bekom en hul moontlike CO_2 fikserende weë was verkry deur middle van die Metacyc databasis. Die CO_2 fikserende weë van belang was die Calvin siklus, die reduktiewe asetiel Ko-ensiem A en reduktiewe sitroen-suur siklusse.

T. scotoductus SA-01 en *E. limosum* was verbasend in staat om lewensvatbaar en metabolies aktief te bly, selfs by 100 bar druk en 100% CO_2. Hierdie vinding was nog nooit van tevore in literatuur berig nie. *Geobacillus* sp. GE-7 en *Geobacillus* sp. A12 was egter nie in staat om lewensvatbaar te bly toe die druk verhoog was van 2 bar na 20 bar of hoër nie.

Die uitsette van hierdie studie toon dat die interaksies tussen superkritiese CO_2 en die ondergrondse biome ook word as biogeochemiese sirkulering oorweeg moet word. Hierdie ondergrondse interaksies is egter steeds relatief onbekend en die beskikbaarheid van interaktiewe metabolise weë dui aan dat die ondergrondse gemeenskappe kan oorleef en reageer met die substraat verskaf.

Sleutelwoorde: Koolstofsekwestrasie, Superkritiese CO_2, Druk, Metaboliese weë, diep-myn mikroörganismes, *Eubacterium limosum*.