ANAEROBIC ETHANOL PRODUCTION USING BRAN AS A SUBSTRATE

By

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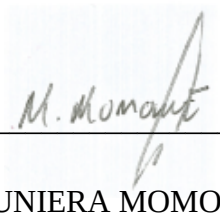
DECLARATION

I declare that this is my own unaided work. It is being submitted for the degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



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ABSTRACT

Biofuels have been exploited as a substitute for non-renewable fossil fuels by humankind for several decades. The source has been mainly plants that are also used to make other important economic products. It is therefore important to investigate other less costly methods of producing this important environmentally friendly fuel. The purpose and focus of this research were threefold; to isolate a thermophilic and mesophilic (control) mixed bacterial consortium that degrades lignocellulose efficiently and produces ethanol, to run an anaerobic fluidised bed bioreactor (AFBR) that produces ethanol and to use molecular methods to identify the bacterial species making up the consortia. Winogradsky columns were used as enrichment culture, and a microaerophilic culture technique was employed to isolate cultures that took from 5 days for complete filter paper degradation (mesophilic and thermophilic). Thermophilic and mesophilic bacterial consortia were constructed from a few of the fastest degrading cultures. The mesophilic consortium isolated was run in an AFBR. The thermophilic consortium (65°C) was obtained from freshly collected elephant, impala, eland and kudu dung collected at the Johannesburg zoo was mixed and inoculated into endo medium and incubated at 65°C. The AFBR was optimised and operated under mesophilic and thermophilic conditions by obtaining a steady reduction in the settled bed volume, indicating cellulolytic degradation of the fluidised bran bed and ethanol production. The population structure of the bacterial consortia was determined using molecular techniques: polymerase chain reaction amplification of ribosomal rDNA using universal markers and DGGE was used to separate species into separate bands (species), indicating the similarity and differences in the population structures in the thermophilic, mesophilic and reactor samples. The result of this study show that the Winogradsky column as well as termites can be exploited as a source bacteria consortia for ethanol production.

DEDICATION

In memory of my grandmothers; Amina Jappie and Sukayna Jardine.

ACKNOWLEDGEMENTS

I would like to acknowledge my family for their support and faith in me. Further I would like to thank my supervisors, friend and co-workers in the lab. I would like to thank the NRF for providing funding, Phumlane Masilela for continuing to collect bioreactor data, and the third-year biotechnology students (2003) for collecting and assembling the Winogradsky columns used for sampling. I would also like to thank the biotechnology Department at the ARC Irene for their advice on running reactors.

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LIST OF ABBREVIATIONS

AFBR	Anaerobic fluidised bed bioreactor
PCR	Polymerase chain reaction
DGGE	Differential gradient gel electrophoresis
$_{dd}H_20$	Double distilled water
bp	Base pairs
GtC	Gigatons carbon
GW	Gigawatts
ATCC	American Type Culture Collection
PV	Photovoltaic
UHP	Ultra-high purity gas

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 The Climate Problem

The global temperature shift is believed to be caused by the increased emission of greenhouse gases (including carbon dioxide, nitrous compounds and methane) (Madsen, 2007). The net impact of global warming so far has been moderate but is likely to become markedly negative with large scale consequences by the end of the century. The environmental effects of the use of fossil fuels have become widely acknowledged. Extreme weather conditions have caused heat waves, forest fires and heavy precipitation more frequently than ever before (Hoffert *et al*., 1998). The more dramatic impacts of climate change, such as the shutdown of the ocean's thermohaline circulation (which is responsible for transporting heat from the equator to the polar regions) and deterioration of the West Antarctic Ice Sheet can be avoided, but society needs to act urgently (Socolow *et al*., 2004).

The United Nations framework convention on climate change was inaugurated on 16 February 2005. The Kyoto Protocol is an amendment to the United Nations framework convention on climate change (Wellington and Bradley, 2006). Countries that ratified this protocol have pledged to reduce their CO_2 emissions and five other greenhouse gases or employ emissions trading if they sustain or increase emissions of these gases (Hoffert, 2004).

The European Union (EU) has set a target of restricting the global temperature rise to 2°C as opposed to preindustrial levels, of which 0.8% has already transpired, and another 0.5% is unavoidable (Pacala and Socolow, 1998). A temperature change of 2% is typically associated with a CO_2 concentration of 400-500ppm by volume. The level of January 2007 was 383 parts per million (ppm) and rising by 2ppm each

year. Hence to circumvent a prospective contravention of the 2% target, carbon dioxide levels would have to be stabilised very soon (Pacala and Socolow, 2004). The world needs to act immediately to establish low-carbon energy technologies and upgrade natural carbon sinks (naturally removes carbon, e.g., the rainforest removes carbon in the form of carbon dioxide to avoid a doubling of carbon dioxide and dramatic climate change (Pacala and Socolow, 2004). As a step to solving this problem, the addition of ethanol to petroleum instead of methyl tert-butyl ether (MTBE), which curtails carbon monoxide (CO) emission and smog-producing hydrocarbons that, has widely been established in the past few years. The demand for bioethanol is, therefore, on the increase (Zaldivar, Nielsen and Olsson, 2001).

1.1.2 Global Energy Crisis

The worldwide energy demand continues to grow as the global population spirals. As a consequence, research and development of novel power that can eliminate the need for fossil fuels have grown. The factors responsible are the decrease in fossil fuel reserves, fluctuation of crude oil prices, unstable and uncertain fossil fuel sources, and the localisation of fossil fuel resources and concern over the environment and global climate change in terms of greenhouse gas emissions and market-related geopolitical issues. (Himmel *et al.*, 2007: Ziniviev *et al.*, 2010).

At present, fossil fuel-derived energy resources account for 79% of the world's energy consumption, nuclear power provides 7%, and renewable energy supplies the remainder. The global energy requirement is estimated to grow by more than 50% by 2025, primarily from rapidly developing nations (Madsen, 2007).

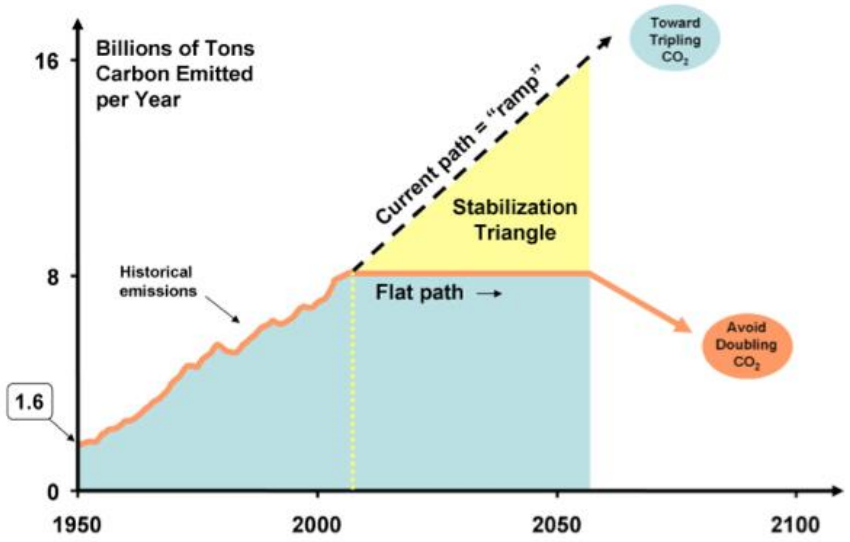
1.1.3 Alternative Fuel Sources

While most existent renewable energy options (e.g., wind, tidal energy, solar, geo-energy and hydro-energy) are suited to the production of electricity, more than a half of the current energy consumption relies on liquid fossil fuels. It is clear though that any alternative energy source must be clean (carbon negative) and sustainable (Pacala and Socolow, 2004). There are numerous approaches on how to address

climate change. The more difficult endeavour is to determine if a mixture of solutions will reduce greenhouse gas emissions on the degree needed to mitigate disastrous climate change impacts (Socolow *et al.,* 2004).

The most pragmatic framework for consideration of solutions to the climate change predicament was the path-breaking August 2004 *Science* paper by Socolow and Pacala who conceived of the concept of "stabilization wedges" shown in Figure 1.1, to demonstrate the nature and scale of enterprises that would be prescribed to stabilise atmospheric conditions (Hoffert, 2002). The idea is elegant and straightforward. The world must reduce emissions by about seven gigatons of carbon to balance emissions in the next 50 years. Socolow and Pacala identify 15 stabilisation wedges, listed in Table 1.1. Each "wedge" correlates to one gigaton/year of the global reduction in carbon by the year 2050. Ideally, seven wedges would be needed to set the maximum CO_2 concentration to below 500 ppm (Figure1.1 a) and in that attain climate stabilisation. It requires a reasonably straightforward calculation to propose practices that can contribute one wedge. For example, a reduction in fossil fuel usage from 30 mpg to 60 mpg for 2 billion produces one wedge. Figure 1.1 b and c show several presumptive wedges to indicate that climate stabilisation can be attained just by using commercially available technologies (Pacala and Socolow, 2004). The change of fossil-based fuels to biofuels, especially bioethanol, would be a compulsory wedge in any CO_2 mitigating strategy (Figure 1.1 c and Table 1.1)

Some of the more radical avenues to reducing the greenhouse effect include carbon sequestration, orbital solar shades, geoengineering, solar power satellites, superconducting global electric grids and population control (Hoffert *et al.,* 2002). The sustainability of global energy with continued economic growth will necessitate, cost-effective and innovative technologies that are carbon-emission-neutral, can provide further tens of terawatts of primary energy in the coming years can only be secured through an integration of various renewable energy products. (Hoffert *et al.,* 2002).



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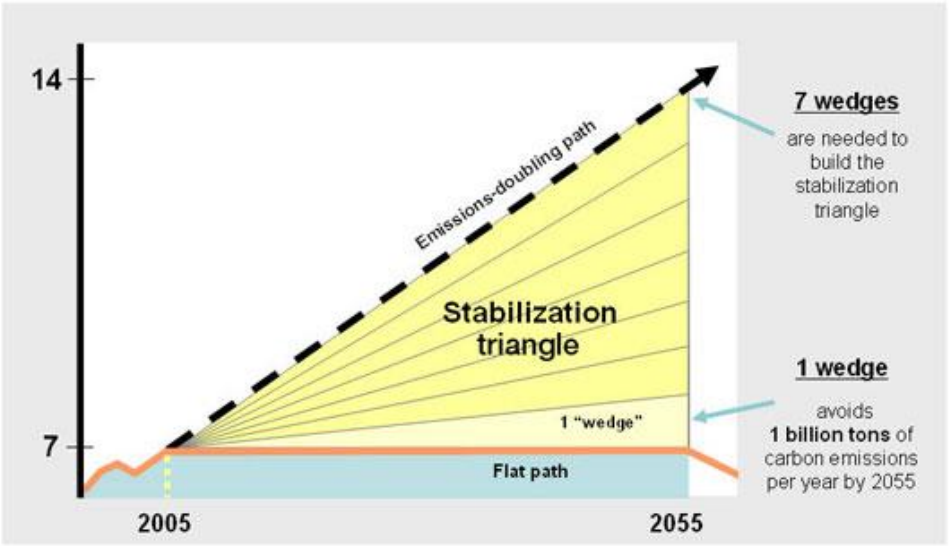

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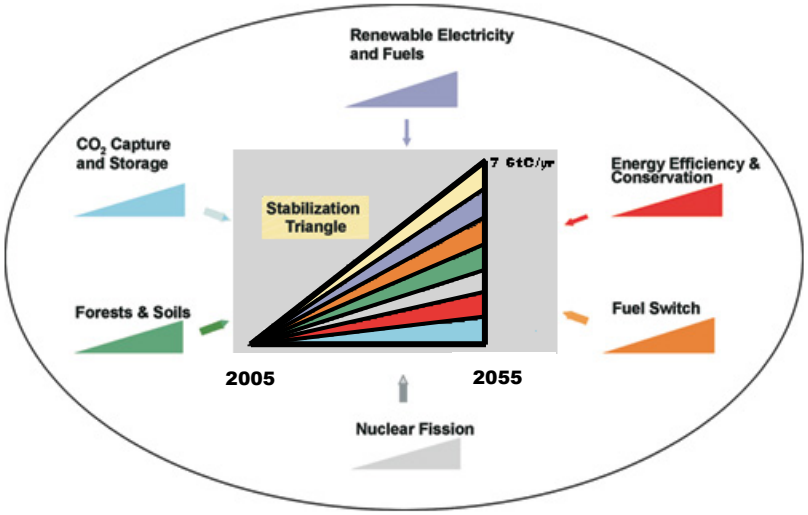


(c)

Figure 1.1: **The "Stabilization Wedges" Concept**. Illustrates the scale of emissions cuts required in the future and provides a standard unit for comparing the carbon-mitigating capacities of various energy and storage technologies.
(a) Historical carbon emissions with two potential future pathways for our currently predicted path (dotted black line) will probably result in at least a tripling of atmospheric CO_2 relative to its preindustrial levels while keeping emissions flat (solid line) would put us on track to avoid a doubling of CO_2. **(b)** The stabilisation triangle can be broken up into seven equal "wedges" that represent activities adequate to reduce one billion tons of carbon per year by 2054. **(c)** The possible examples of wedges that could be used to make up a stabilisation triangle are summarised.

Source: (Socolow *et al.*, 2004; Pacala, 2009)

Table 1.1: Potential Stabilization Wedges: Strategies available to reduce the carbon emission rate in 2054 by 1 GtC/year (total 25 GtC)

	Option	The effort required relative to 14 GtC/year
Energy efficiency and conservation	1. Efficient vehicles	Increase fuel economy for 2 billion cars from 30 to 60 mpg
	2. Reduced use of vehicles	Improve urban design to reduce miles driven from 10,000 to 5,000 miles per year for 2 billion vehicles
	3. Efficient buildings	Reduce energy consumption by 25%
	4 Improve the efficiency of coal plants	Produce twice the current coal power output at 60% instead of 40%
Fuel shift	5. Replace coal with gas	Replace 1,400 GW of coal power plants with natural gas
Nuclear fusion	6. Nuclear power for coal power	Displace 700 GW of coal power with nuclear
Forests and agricultural soils	7. Stop deforestation, start reforestation, afforestation, and new plantations.	Re-establish 300 million hectares of new tree plantations
	8. Conservation Tillage	Apply to all cropland (10 times the current usage)
CO_2 Capture and Storage (CCS)	9. Capture CO_2 coal plant	Capture and store carbon emitted from 800 GW of new coal plants
	10. Capture H_2 plant at a coal plant	Capture and reuse hydrogen
	11. Capture CO_2 at a coal-to-synfuels plant	Capture and store carbon from coal to syn-fuels conversion at 30 million barrels per day (4,800,000 m^3/d)
Renewable electricity and fuels	12. Wind power for coal power	Add 2 million 1 megawatt windmills (50 times the current capacity),
	13. PV power for coal power	Displace 700 GW of coal with 2,000 GW (peak) solar energy (700 times the current capacity)
	14. Wind H_2 for petroleum	Produce hydrogen in a fuel-cell car for gasoline in a hybrid vehicle from 4 million megawatt windmills
	15. Biomass fuel for fossil fuel	**Use biomass to make fuel to displace oil (100 times the current capacity)**

GtC:Gigatons carbon, GW:Gigawatts, PV:hotovoltaic, Source: (Pacala and Socolow, 2004)

Table 1.2: Categories of Renewable Energy Conversion Technologies

Technology	Energy Product	Application
Biomass energy		
Combustion (domestic scale)	Heat (cooking, space heating)	Widely applied: improved technologies available
Combustion (industrial scale)	Process heat, steam, electricity	Widely applied; potential for improvement
Gasification/power production	Electricity, heat(CHP)	Demonstration phase
Gasification/fuel production	Hydrocarbons, methanol, H_2	Development phase
Hydrolysis and fermentation	**Bioethanol**	**Commercially applied for sugar/starch crops; production from wood under development**
Pyrolysis/production of liquid fuels	Bio-oils	Pilot phase; some technical barriers
Pyrolysis/production of solid fuels	Charcoal	Widely applied; wide range of efficiencies
Extraction	Biodiesel	Applied; relatively expensive
Digestion	Biogas	Commercially applied
Wind energy		
Water pumping and battery charging	Movement, power	Small wind machines; widely applied
Onshore wind turbines	Electricity	Widely applied commercially
Offshore wind turbines	Electricity	Development and demonstration phase
Solar energy		
Photovoltaic solar energy conversion	Electricity	Widely applied; rather expensive; further development needed
Solar thermal electricity	Heat, steam, electricity	Demonstrated; further development needed
Low-temperature solar energy use	Heat (water, cooking, drying) and cold	Solar collectors commercially applied; solar cookers widely used in some regions; solar drying demonstrated and applied
Passive solar energy use	Heat, cold, light, ventilation	Demonstrations and applications; no active parts
Artificial photosynthesis	H_2 or hydrogen-rich fuels	Fundamental and applied research
Hydropower	Power, electricity	Commercially applied; small and large scale applications
Geothermal energy	Heat, steam, electricity	Commercially applied
Marine energy		
Tidal energy	Electricity	Applied; relatively expensive
Wave energy	Electricity	Research, development and demonstration phase
Current energy	Electricity	Research and development phase
Ocean thermal energy conversion	Heat, electricity	Research, development and demonstration phase
Salinity gradient/osmotic energy	Electricity	Theoretical option
Marine biomass production	Fuels	Research and development phase

(Turkenberg *et al.*, 2000)

1.1.4 Renewable Fuels

The primary requirement for energy is thus that it be carbon neutral, sustainable and renewable. Renewable energy uses natural resources, which are naturally replenished (Pacala and Socolow, 2004). These renewable energy sources have the potential to reverse the harmful effects of fossil fuels on the environment (Turkenberg, 2004). Renewable energy can provide the current global energy demand many times, and their potential is massive (Turkenberg, 2004). They can increase diversity in energy supply markets and secure sustainable energy supplies in the long term. There are many renewable technologies. Most are still at an early stage of development and not technologically mature even though they are often commercially available. Continuing research, development and demonstration efforts are required. The current state of these technologies is shown in Table 1.2. Also, few renewable energy technologies can compete with the cost of conventional fuels. Substantial cost reductions will be required for further technology development, marketing and mass production (Turkenberg, 2004).

1.1.5 Transportation Energy

Electricity can be produced from all renewable energy sources, but only a few of them can produce gaseous and liquid fuels as well as heat directly (Turkenburg, 2000). Several fuels can be produced from biomass feedstocks, shown in Table 1,2, including gaseous fuels, such as methane and hydrogen, and liquid fuels, such as ethanol, biodiesel, Fischer-Tropsch diesel and methanol. The term biofuel refers to gaseous or liquid fuels used in the transport sector that is primarily produced from biomass (Demirbas, 2008).

The primary liquid biofuels considered to fall into the following categories: bio-alcohols; biodiesels and vegetable oils; and bio-synthetic and bio-crude oils (Demirbas, 2008).

1.1.6 Bioethanol

Ethanol is perhaps the oldest product attained through traditional biotechnology. Henry Ford planned for cars fueled by bioethanol (ethanol from biomass) in his initial design for model T's that utilised "farm ethanol" made from corn (Zaldivar *et a.l*, 2001).

The discovery of cheap fossil fuels, however, quickly dominated the market up to the 1970s 'oil crisis', which resulted in the realisation that the world's oil supply is not infinite (Demain *et al.*, 2005). This awareness initiated the quest for economic alternatives to fossil fuels to curtail the dependence on imported non-renewable fossil fuels, generating initiatives such as the National Alcohol program in Brazil and the "gasohol" program in the USA. As oil prices depreciated over the following decades, however, so did interest and demand for bioethanol.

Excessive consumption of fossil fuels for transportation has directly led to global environmental deterioration effects such as climate change, greenhouse effect, ozone depletion, and acid rain, which has dramatically contributed to generating high levels of pollution and smog, especially in metropolitan areas. Also, the escalation in fossil fuel prices and instability of the market has finally led to the realisation that the world's oil supply is going to run out, so the pursuit of alternative renewable transportation fuels has been re-ignited.

In response, many countries have instituted extensive research programs to develop renewable and sustainable energy sources that can generate bioethanol. The United States Department of Energy (DOE) Office of the Biomass Program has set the target for supplying 30% of the 2004 petroleum consumption with biofuels by 2030, which translates to about 60 billion gallons per year (Himmel *et al.*, 2007). The European Union (EU) envisages a future where a quarter of its transportation fuels will arise from biofuels by 2030 (Himmel *et al.*, 2007).

Bioethanol as a transportation fuel has already been instituted as a sustainable biofuel economy in Brazil. It has been introduced on a large scale in the US and

some European countries and is anticipated to be one of the leading renewable fuels in the transport sector within the coming decades. Figure 1.2 shows the increase in global fuel ethanol production.

Table 1.3: Benefits and Risks of Biofuels

Advantages	Risks
• Crude oil reserves extended (intergenerational benefit) • Benefits from increased expenditure on fuels to agriculture - Job creation - Farmers - Agribusiness - Rural poor • Sustainable development - Job creation • Reduces pollution • Low nett CO_2 release • Co-products	• Poor results for nitrous oxide emissions • Competition for land with food and fibre production • Transportation of large volumes of low energy density biomass feedstocks • Impacts of processing (stillage) • Hedge against high fuel prices • Water requirements

(Turkenberg *et al.*, 2000)

1.1.7 Performance of Fuel Ethanol

Ethanol is an excellent alternative to fossil fuel, especially as a transportation fuel (Demain *et al.*, 2005). Ethanol fermentation offers a favourable trade balance and would be produced locally, providing enhanced energy security (Lynd, 1996).

Ethanol is substantially less harmful to people than petroleum or even methanol (Demain *et al.*, 2005). Bioethanol can be used as a liquid fuel in four ways: low-level blends (≤ 22% ethanol in petroleum), high-level blends (≥85% ethanol), neat (with the addition of ≤ 20% water) or as an additive, i.e., ethyl-tert butyl ether (ETBE) instead of MTBE. Low-level ethanol blends can be used in automobiles with unmodified internal combustion engines, flexi-fuel internal combustion engines or compression ignited engines. It can be blended with petrol or used undiluted in dedicated engines (Lynd, 1996).

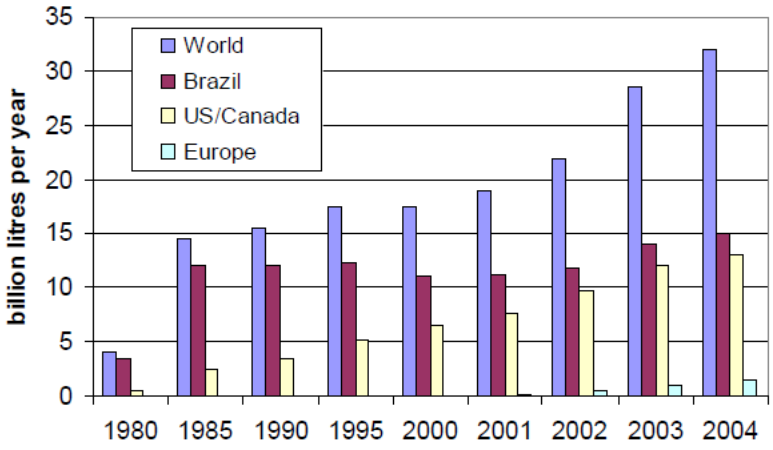
Table1.4: Summary of Ethanol Fuel Properties concerning Fuel Performance

Fuel property	Ethanol to gasoline ratio	Qualitative impact(s)	Thermal efficiency impact (%)
Energy density	0.65-0.69	Lower miles/gallon Larger tank and heavier vehicle for the same range	- 0.9
Heat of vaporisation	2.3	A greater mass of air enters the cylinder Increased power Decreased cooling system duty	—
Lower flame temperature	0.976	Higher efficiency in an optimised engine	—
The relative volume of combustion products	1.07	Increases the work available from gas expansion	+ 7
Octane number	1.15	Allows increased compression ratio and hence higher power and efficiency	+ 6-10

Source: (Lynd, 1989)

Ethanol produces a lower net CO_2. It burns cleaner than regular petroleum, its photochemical reactivity and its combustion products are small; therefore, moderate levels of compounds that produce smog, carbon monoxide, sulfur and oxides of nitrogen are produced by its combustion (Agarwal, 2007). It has a high heat of vaporisation, lower flame temperature and high-octane number (Table 1.3). Ethanol is economically and technically excellent as a fuel for an internal combustion engine (ICE). It is stable and is thus ideal for safe storage and distribution (Lynd, 1996). There are many advantages to replacing fuel ethanol for fossil fuels, but there some disadvantages listed in Table 1.5

Liquid biofuels are predominantly used to fuel automobiles but can be used directly in fuel cells to produce electricity and are, therefore, an exceptional fuel for prospective advanced flexi-fuel hybrid vehicles (Demain *et al.*, 2005). Fuel cells have the advantage of offering high efficiency and very low emissions.



Source: (Schieder, 2005)

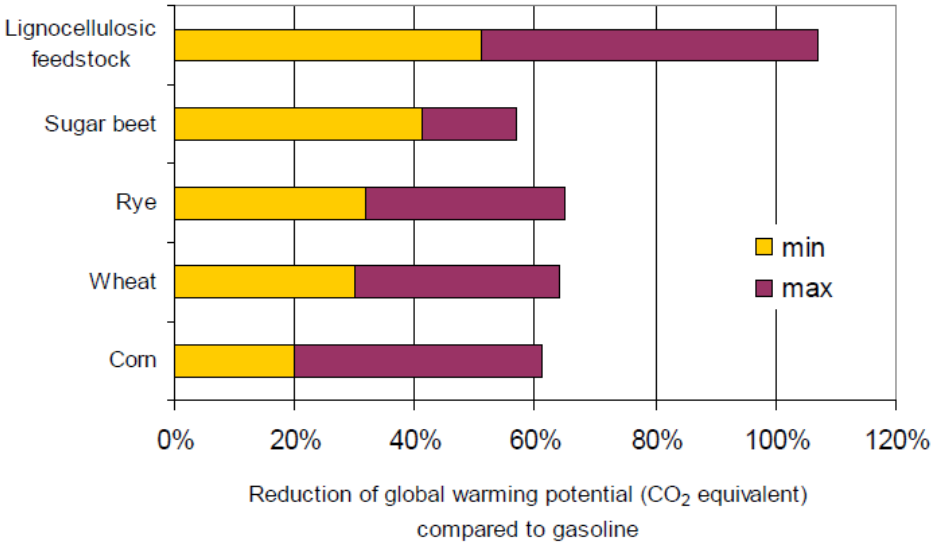
Figure 1.2: Production of Fuel Ethanol from 1980 to 2004

1.1.8 Ethanol Fuel Cells

Biohydrogen can be converted to electricity in a fuel cell. It can either be stored on an automobile or generated in situ by an organic fuel reformer. Methanol has chiefly been investigated to use in fuel cells because it readily dissociates into synthesis gas (H_2 and CO) by steam reforming at moderate temperatures with the aid of appropriate catalysts. No catalyst effective for the steam reforming of ethanol is known; therefore, an alternative technology is required. i.e., auto-thermal reforming and partial oxidation, which is oxygen-limiting combustion at a higher temperature than is needed for steam reforming, producing H_2, CO and CO_2 as products, is being investigated (Lynd, 1996).

1.1.9 Cellulosic Ethanol

It has long been established that lignocellulosic biomass is a prospective sustainable source of mixed sugars for fermentation to biofuels and biomaterials (in a biorefinery), as it is renewable, abundant and reasonably low-cost (Himmel *et al.*, 2007). Lignocellulose is estimated to account for 50% of the global biomass (10-50 billion tonnes). The possible contribution of these sources of biomass feedstocks is far higher since competing uses for biomass are limited, and the demand for co-products is expected to be compatible with the fuel markets (Zaldivar *et a.l*, 2001).



Source: (Schieder, 2005)

Figure 1.3: Well-to-Wheel Greenhouse Gas Emissions. Fermentation fuel ethanol from various feedstock crops: estimated reduction of global warming potential compared to petroleum. The baseline (0%) refers to petroleum.

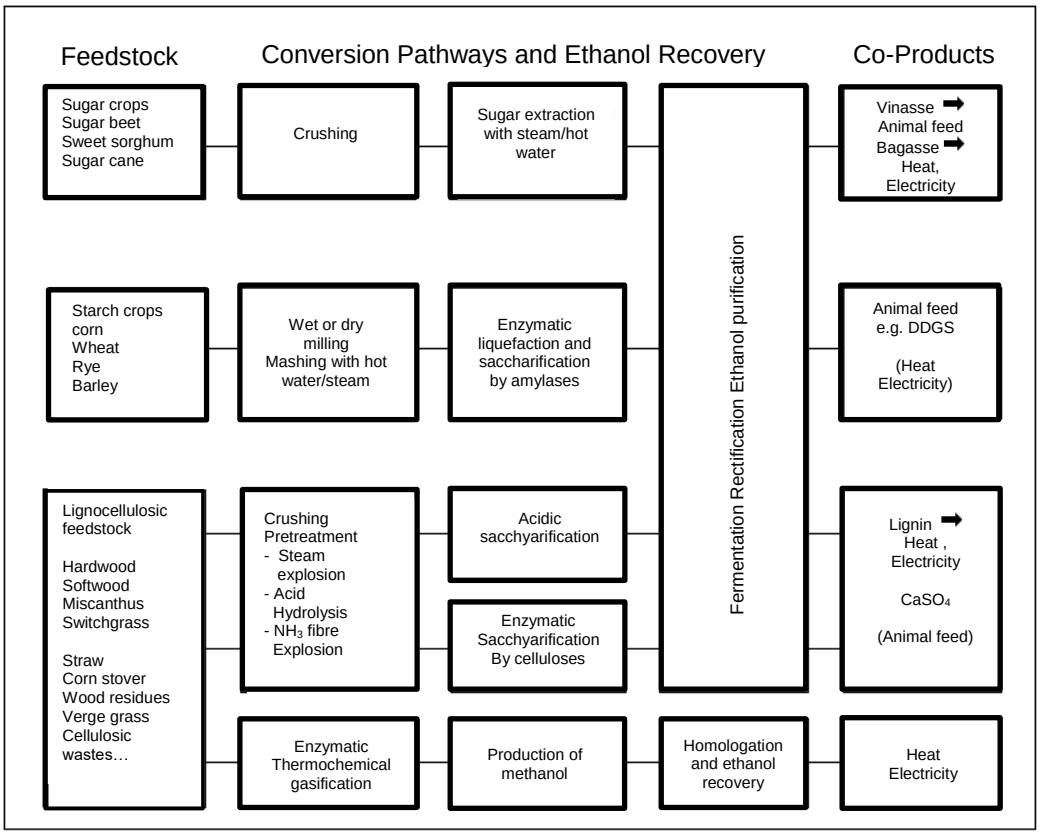
Biomass energy, especially bioethanol, can play a significant role in reducing greenhouse gas emissions. Biofuels from lignocellulose generate low nett greenhouse gas emissions since the CO_2 that arises would initially have been absorbed from the air during photosynthetic growth (Clifford, 2018). Figure 1.3 shows the reduction in CO_2 emissions when using lignocellulose derived ethanol instead of petroleum.

Table 1.5: Use of Ethanol-Based Fuels and Preferred Feedstock Crops in Selected Countries

Country	Typical Fuel blends used	Preferred feedstocks	Estimated fuel ethanol production in 2003 (million litres)
Brazil	blends up to E26	sugar cane	± 14 000
USA	E10 and E85	corn	10 600
Canada	E10	wheat	n.a.
France	ETBE	sugar beet, wheat	125
Spain	ETBE	wheat, barley	225
Sweden	E10, E85, E95	wheat	50
Germany	ETBE	wheat, rye	start-up of first plants in 2004

Source: (Schieder, 2005)

Biofuel sources are more evenly dispersed on earth than fossil fuels, so sources of energy feedstocks will be more prominently be domestic and providing a more secure supply. The use of lignocellulose minimises the competition between land use for human and animal food or the use of arable land used to grow feedstocks energy feedstock production. The raw material is less expensive than conventional agricultural feedstock (corn, sugarcane, sugar beet, etc.) and can be produced with less requirement for fertilisers, energy and pesticides. Biofuels may also create employment in rural areas (Schieder, 2005).



Source: (Schieder, 2005)

Figure 1.4: Production Pathways of Bioethanol from Various Feedstocks.

Currently, ethanol for the fuel market utilises crops grown exclusively to used for biofuel production, such as sugar cane juice in Brazil (National Alcohol Program), corn-starch in the USA (Gasohol) (Lynd, 1996), and sugar beet in France (Zaldivar *et al.*, 2001). Table 1.4 gives a global breakdown of ethanol production from starch or sugar-based crops. However, this raw material base, which also must be used

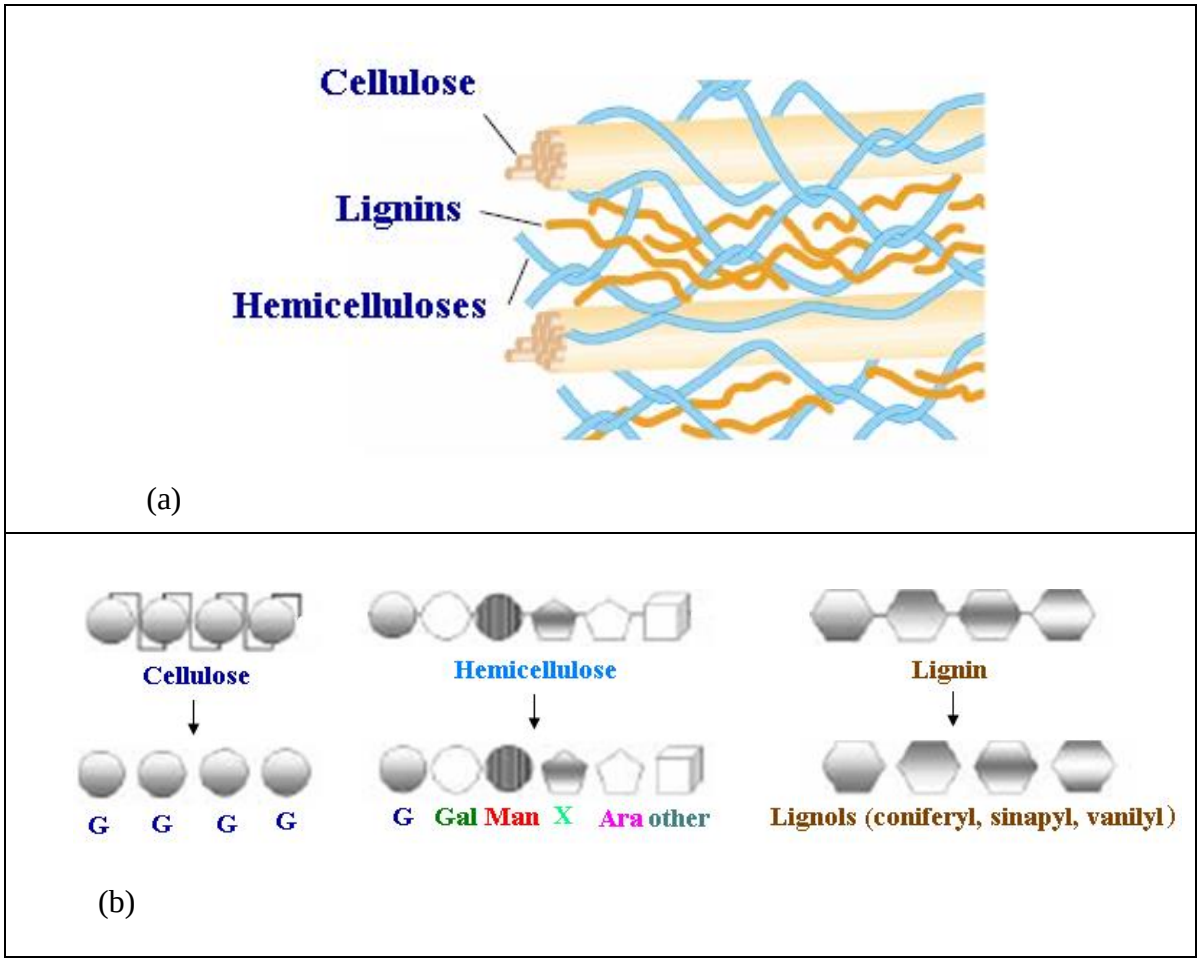
for animal feed and human needs, will not be adequate to meet the escalating demand for bioethanol as a vehicle fuel; and the reduction of greenhouse gases resulting from use of sugar or starch is not as high as desired. Also, the cost of raw materials may reach 40% of the price of ethanol; therefore, recent efforts have concentrated on utilising biomass (lignocellulose) feedstocks, such as agricultural and forest residues as well as dedicated crops, for the production of bioethanol (Wyman, 1999; Mukherjee, 2017). The production pathways for bioethanol is shown in figure 1.4. It shows the three different categories of feedstock, the processes used in conversion to bioethanol and the various by-products produced.

1.1.10 Structure of Lignocellulose

Lignocellulosic biomass is a sustainable, renewable resource. It is a potential source of fuel, energy and chemicals (Wand *et al*., 2011). It is produced by the process of photosynthesis, which captures CO_2 from the atmosphere and stores it chemically in plant biomass. The amount of CO_2 released upon combustion of biomass is equal to the amount initially trapped during photosynthesis (Turkenburg, 2000; Socolow *et al.*, 2004).

Lignocellulose is a complex material comprised of three representative organic fractions with representative compositions as follows: 35-60% cellulose, 20-35% hemicellulose, and 12-20% lignin. Lignocellulose is associated with hemicellulose making it difficult to hydrolyze because it acts as an amorphous cementing agent, and lignin acts a barrier effectively sealing the cellulose (Hazelwood and Gilbert, 1993).

Cellulose is a polymer purely made up of glucose (six-carbon sugar), a or hexose (five-carbon sugar), but crystalline microfibrils are insoluble, making it highly resistant to hydrolysis (Beguine, 1994; Mukherjee, 2017). It comprises long chains of glucose sugars, joined by beta linkages. Linear cellulose chains are arranged in parallel held together by comprehensive hydrogen bonds that give it an extremely ordered, tightly packed structure, impeding rapid breakdown to glucose (Demain *et al.*, 2005; Lynd, 1996)



Source: (Zaldivar *et al.*, 2001) and (Boudet *et al*., 2003)

Figure 1.5: Structure of Lignocellulose. (a) Schematic Representation of the Lignocellulose. (b) Components of Lignocellulose

Hemicellulose is a polymer made up of an amorphous chain of a mixture of sugars, usually including arabinose, galactose, glucose, mannose, and xylose, as well as smaller amounts of a few other compounds, such as acetic acid. The composition and proportion of different sugars comprising hemicellulose depend on the type of plant material. The dominant constituent in plants other than softwoods, is xylose, a five-carbon sugar or pentose (Lynd, 1996). Hemicellulose chains are more easily broken down to form their component sugars than is cellulose (Wyman, 1999).

Lignin is not a sugar-based structure. It is a heterogeneous polymer based on a phenyl propylene backbone. These subunits are attached to ether or C-C linkages, which are covalently linked to hemicellulose (Lynd, 1996). Cellulose and hemicellulose can be converted into ethanol, but no known microorganism can use

lignin to produce ethanol as a byproduct. Residual lignin could be sold as adhesive formulations, burned as fuel or converted to steam and electricity (Beguin and Aubert, 1994; Wyman, 1999).

1.1.11 Biomass Feedstocks

Cellulosic biomass for use in bioethanol production can come from either waste material such as agricultural waste, forestry byproducts or as crops, etc. are grown primarily for fuel production (energy crops). The first feedstocks to be used for fuel ethanol production are most likely to be wasted because of their lower cost than dedicated energy crops. However, the amounts of ethanol required to displace petroleum on a large scale will, in time, necessitate the use of dedicated feedstocks such as short-rotation woody crops (e.g., poplar, willow) and perennial herbaceous crops (e.g., switchgrass) as well (Lynd, 1996).

The use of waste materials for fuel ethanol production has no land-use requirements and raises fewer environmental issues. Converting waste into fuel ethanol may even save land by reducing the flow of waste material to landfills. Also, minimal resources are required other than for collection. Concerns about energy crop production include issues of environmental impacts that are not yet completely understood (Lynd, 1996).

Lignocellulosic biomass for fuel ethanol production can be divided into six main groups: crop residues, cellulose wastes (waste office paper, newsprint, recycled paper sludge), corn stover, cane bagasse, wheat straw, rice straw, rice hulls, barley straw, sweet sorghum bagasse, olive stones and pulp, hardwood, softwood, herbaceous biomass and municipal solid wastes (MSW) (Sanchez and Cardona, 2007).

Wheat bran is a by-product of the wheat milling industry. Wheat milling is an almost ubiquitous global activity. It is estimated that the wheat milling industry globally produces in the order of 96 million tonnes of wheat feed (unrefined wheat bran). It is mostly pelleted and sold as animal feed. The market for wheat feed and

wheat bran is restricted, and the wheat milling industry is continually generating huge surpluses. Alternative uses are desirable, especially if it leads to renewable sources of energy via non-fossil routes (Hawkes et al., 2008). It may present a unique opportunity for the utilisation of surplus Agricultural wastes such as surplus wheat bran as a feedstock for the generation of biofuels as well as coproducts in a biorefinery. Multiple end products make the process attractive and more economically viable (Angelidaki, 2007; Raposo et al., 2016).

Wheat is imported to, as South Africa does not produce enough to fulfil its requirements. For this reason, most bran produced is used as animal feed produced for bioethanol production. Other crop residues such as maize stover and sugar and sugar cane bagasse have been considered as feedstock for biofuel production (Mutenure, 2016)

Table 1.6: Comparison of Pretreatment Processes

Feature	Process			
	Dilute acid	Steam explosion	AFEX	Liquid hot water
Reactive fibre	Yes	Yes	Yes	Yes
Particle size reduction required	Yes	-	-	-
Hydrolyzate inhibitory	Yes	Yes	-	Slightly
Pentose recovery	Moderate	Low	High	High
Low-cost materials of construction	-	Yes	Yes	Yes
Production of process residues	Yes	-	-	-
Potential for process simplicity	Moderate	High	Moderate	High
Effectiveness at low moisture contents	Moderate	High	V high	Not known

(Mosier *et al.*, 2005), (Lynd *et al.*, 2002)

1.1.12 Biomass Pretreatment

Lignocellulose is insoluble and highly resistant to enzymatic hydrolysis (Beguin and Aubert, 1994) because: it is associated with hemicelluloses, a lignin seal surrounds it, and cellulose has a highly ordered, tightly packed crystalline structure (Demain *et a.l*, 2005). Pretreatment aims to remove the lignin seal, thereby

increasing the exposed surface area of cellulose, solubilising the hemicellulose matrix, impeding crystallinity and expanding pore volume. Hemicelluloses are hydrolysed into simple 5-C and 6-C carbon sugars and short-chain oligosaccharides, which can be further hydrolysed and used for fermentation. Pretreatment additionally removes hemicellulose from the microfibrils to expose the crystalline cellulose core to hydrolysis by cellulase enzymes. Also, pretreatment generally decreases the overall recalcitrance lignocellulose making it more accessible.

Substantial research has gone into advancing the development of strategies for converting cellulose to pentose and hexose sugars. Heat and chemical pretreatment of biomass is a critical process to materials more accessible to enzymatic digestion. A commonly used pretreatment uses dilute sulfuric acid at 140 to 200°C, which increases the accessibly of cellulose to hydrolyzing enzymes. At moderate severities, yields of sugars produced are lower, i.e. only 60 to 70%.

AFEX Ammonia fibre expansion pretreatment is based on alkaline explosive decompression. This process keeps hemicellulose intact but leaves the remaining cell walls substantially more accessible enzyme hydrolysis. Organic solvent extraction has successfully been utilised.

Several criteria characterise an effective pretreatment. It avoids the need for reducing the size of biomass particles, preserves the pentose (hemicellulose) fractions, limits the formation of degradation products that inhibit the growth of fermentative microorganisms, minimises energy demands and limits cost (Lynd,1996). The results of pretreatment must be balanced against their impact on the cost of the downstream processing steps and the trade-off between operating costs, capital costs, and biomass costs. (Mosier *et al.*, 2005).

1.1.13 Cellulolytic Ethanol Producers

Ethanol is commonly produced as a fermentation end-product, but with most organisms, ethanol is only one of a mixture of fermentation products which commonly includes: lactate, succinate, acetate, formate, H_2 and CO_2. The two main microorganisms that produce ethanol and CO_2 as the only fermentation end-products (homo-ethanolic) are yeasts of the genus *Saccharomyces* and bacteria in the genus *Zymomonas*. For industrial ethanol production, *Saccharomyces* and *Zymomonas* have some disadvantages: they are not thermophilic or thermotolerant, and they have a restricted substrate range (Levett, 1990; Lynd, 2002).

Much work has focused on cellulose hydrolysis by the cellulolytic enzymes produced by the fungus *Trichoderma reesi*, followed by the conversion of the sugars generated, to ethanol by the yeast *Saccharomyces cerevisiae* (Demain *et al*., 2005). This process is complex and involves coupling the thermodynamic inefficiencies of two separate eukaryotic organisms. The process is mesophilic and aerobic, which means that a considerable input of energy is required for distillation and oxygenation (Zeikus, 1979; Lynd).

Thermophiles have some potential advantages: no cooling is required during vigorous fermentation; thermophiles tend to have rapid growth rates; it may be possible to distil ethanol directly from fermentations particularly if a slight vacuum is applied (Solowski, 2018).

Organisms must produce a high ethanol yield from glucose, the end product of cellulose hydrolysis. All organisms studied so far produce other by-products other than ethanol. Wild type strains of *Clostridium cellobioparum* and *Thermoanaerobacterium brockii* produce less than one mole of ethanol per mole of glucose. Recently, however, mutants of *C. thermocellum* have been isolated that produce greater ethanol yields. *C. thermohydrosulphuricum*, *C. saccharolyticum* and *Thermoanaerobacter ethanolicus* typically produce greater ethanol yields; in the region of 1.8-1.9 moles per mole of glucose have been obtained. These yields depend on strain and growth conditions and can vary greatly. Wild type *T.*

ethanolicus, for example, produces 1.8 moles of ethanol per mole of anhydrous glucose at 0.5% w/v starch but only one mole or less at substrate concentrations ≥ 1% w/v (Solowski, 2018).

High tolerance for high ethanol concentrations is vital to optimising the economic factors of both fermentation and product recovery. *Saccharomyces* and *Zymomonas* generally have a relatively high ethanol tolerance. Strains of *Z. mobilis* have been shown to produce 100-120 g/l of ethanol, while for Saccharomyces 160 g/l or better can be obtained during the production of sake (Japanese rice wine), and 110 g/l is quite common (Lynd, 2002).

The situation with anaerobes, however, is very different, as all seem to be much more sensitive to ethanol. Wild type strains of *C. thermocellum*, for example, are inhibited by 50% by 6 g/l of ethanol, although mutants with enhanced ethanol resistance (50% inhibition at ethanol concentrations of 55 g/l) have been isolated. Similarly, wild type *T. ethanolicus* cannot accumulate more than 4 g/l of ethanol, but mutants producing almost 30 g/l have been isolated. A mutation can be used to improve ethanol tolerance and production (Zaldivar *et al*., 2001).

Table 1.7: Desirable Properties of Ethanol Producing Microorganisms

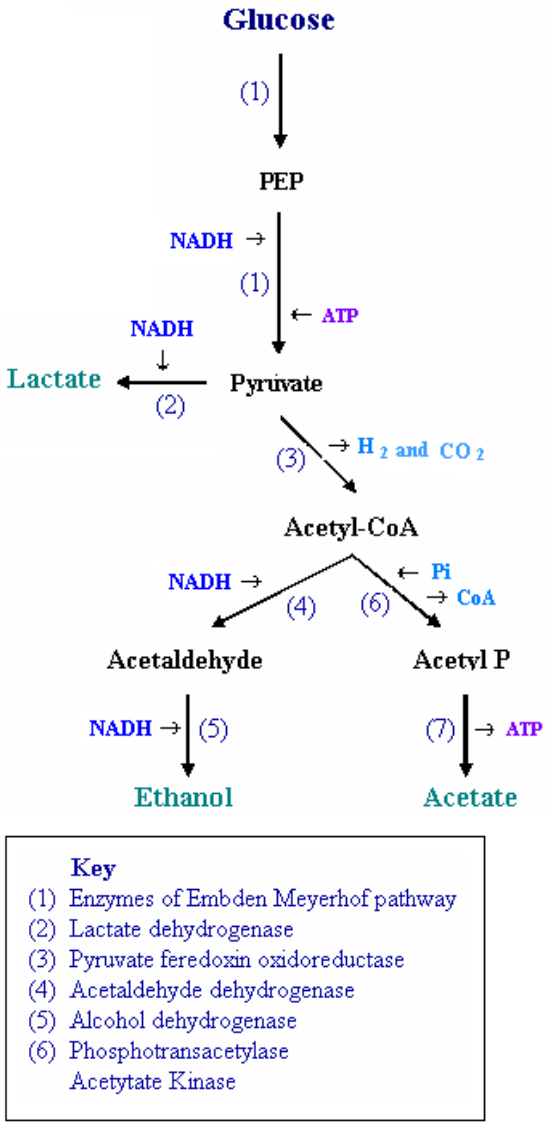
- Tolerance to high ethanol concentration
- Tolerance to high concentrations of other fermentation byproducts
- Tolerance to high substrate concentrations
- Rapid growth and fermentation rates
- Ability to ferment sugars to give high molar yields of ethanol (close to the theoretical maximum of two moles/mole of hexose)
- Thermophilic or thermotolerant
- Capable of fermenting a wide range of saccharides commonly found in biomass or organic wastes, e.g., cellulose, starch, hemicellulose, cellobiose, lactose, galactose, pentoses (especially xylose)
- Capable of simultaneous use of different sugars, i.e. not subject to catabolic repression

Source: (Levett, 1990)

Ethanol has been found to cause alteration in the lipid content of cell membranes of anaerobes such as *C. thermocellum* and *C. thermohydrosulfuricum*. In order yo prevent disruption in the cell membrane may necessitate the growth of thermophiles

below their optimum temperature to obtain high ethanol concentrations (Zaldivar *et al.*, 2001.

One of the potential advantages of anaerobes is their ability to degrade cellulose, hemicellulose and to utilise hexoses, thus enabling the fermentation and hydrolysis of cellulosic biomass in one step.



(Source: Demain *et al.*, 2005)

Figure 1.6: Pathways to Ethanol Production by *Clostridium* species

Clostridium thermocellum utilises only hexoses from cellulose, not the pentoses such as xylose and xylobiose derived from its breakdown of hemicellulose. For this reason, a co-culture with another anaerobic thermophile is required for the conversion of pentose sugars to ethanol. Co-cultures of *C. thermocellum* with

saccharolytic thermophiles such as *C. thermosaccharolyticum, C. thermohydrosulfuricum* (Ng, Ben-Bassat and Zeikus, 1981), *T. ethanolicus and T. brockii* (Lamed and Zeikus, 1980) have been shown to produce more ethanol than *C. thermocellum* alone. This improvement is primarily due to the ability of the second organism to ferment xylose and cellobiose generated by the cellulose-degrading coenzymes of *C. thermocellum* (Wang *et al*., 1983; Levett, 1990).

Despite much research and improvement, ethanol production by thermophilic anaerobes cannot yet compete with conventional organisms. The potential advantages have to be weighed against the low ethanol tolerance and relatively low yields of ethanol obtainable (Levett, 1990).

An anaerobic microbial system that displays low to no acid formation with high ethanol yields and rates of reactivity does not yet exist. Genetic engineering should offer a solution. One approach has been to add cellulolytic genes to a non-cellulolytic ethanol producer such as *Saccharomyces cerevisiae*. (Demain *et al.*, 2005). Table 1.7 is a summary of the desirable properties of ethanol-producing microorganisms.

1.14 The *Clostridium thermocellum* Model

Clostridium thermocellum is a thermophilic cellulolytic anaerobe that can generate ethanol in a one-step bioconversion of cellulose to ethanol (See Table 1) (Demain *et al*., 2005). Bioconversion of cellulose in a single step is a rare property among living organisms.

The bacterium *C. thermocellum* degrades cellulose, with the formation of cellobiose and cellodextrins as primary products. Cellobiose, a disaccharide of two glucose molecules held together by a β-1,4 linkage, can be further utilised by the organism, and the final products are ethanol, acetic acid, lactic acid, hydrogen and carbon dioxide (Zeikus, 1979).

Clostridium thermocellum shows tremendous potential. It is cellulolytic and ethanol-producing, allowing the breakdown of cellulose to monomeric sugars as

well as fermentation in a single step. It is an obligately anaerobic, eliminating the requirement of costly oxygen transfer. It has a low cell growth rate resulting in low bacterial biomass yield, promoting ethanol conversion. Its thermophilic growth conditions are less prone to contamination and promote the recovery and removal of ethanol. Thermophilic lignocellulose degrading enzymes improve protein stability and allowing for the co-culture with other fermenting organisms that produce ethanol and ferment pentose. It has a growth rate on cellulose that is more rapid than mesophilic systems, with ethanol as a primary fermentation product (Lynd, 2001).

1.1.15 Consolidated Bioprocessing

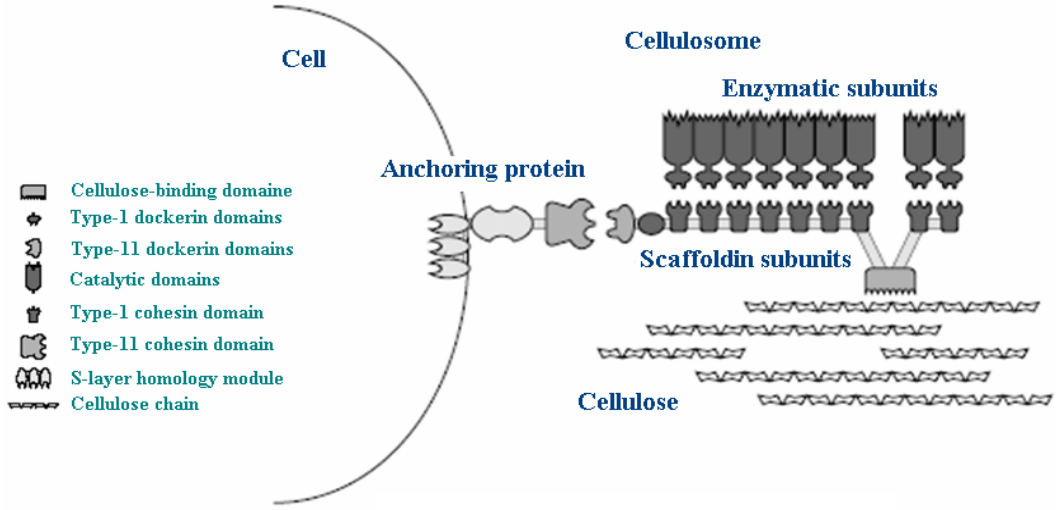
All organisms are known to degrade cellulose efficiently produce a battery of enzymes with different specificities, which act together in synergism (Beguin and Aubert, 1994).

For microorganisms to hydrolyse and metabolise insoluble cellulose, extracellular cellulases must be produced that are free or cell-associated (Lynd *et al*., 2002). The cellulase system of the aerobic, mesophilic, fungus *Trichoderma reesi* comprises three main components: endoglucanases, exoglucanases and β-glucosidases that act synergistically in the hydrolysis of crystalline cellulose (Beguin and Aubert, 1994). *T. reesi* excrete these cellulases in large quantities to mediate cellulose hydrolysis (Lynd *et al.,* 2002).

In contrast, anaerobic microorganisms have evolved a system to break down plant cell walls that involve the formation of a large extracellular enzyme complex called the cellulosome, which consists of a protein scaffoldin with many bound cellulases (Shoham *et al*., 1999). The cellulosome of *C. thermocellum* is composed of: numerous endo-B-gluconases responsible for the degradation of amorphous varieties of cellulose, e.g., TNP-CMC (trinitrophenyl-carboxymethylcellulase) CMC; at least four exoglucanases; a cellobiose phosphorylase that breaks down cellobiose to glucose and glucose-1-phosphatase; a cellodextrin phosphorylase that phosphorylates B-1,4-oligoglucans; and two B-glucosidases that hydrolyze

cellobiose to glucose. *C. thermocellum* also possesses at least six xylanases, two linamirases, two lichenases, and minor activities of B-mannosidase, B-galactosidase and B xylanases (Schwarz, 2001).

The cellulosome, shown in Figure 1.7, mediates the attachment of the bacterium to lignocellulose, which in turn enhances the activity of cellulolytic enzymes (Beguine, 1994). *Clostridium thermocellum* has the largest and most well-characterised cellulosome, which has a very high activity on crystalline (e.g., cotton and Avicell) and amorphous cellulose. Fungal cellulases such as those produced by *Trichoderma reesi*, on the other hand, excrete a high extracellular concentration but has a rather weak activity even on amorphous cellulose and cannot hydrolyse crystalline cellulose without pretreatment (Doi and Kosugi, 2004).

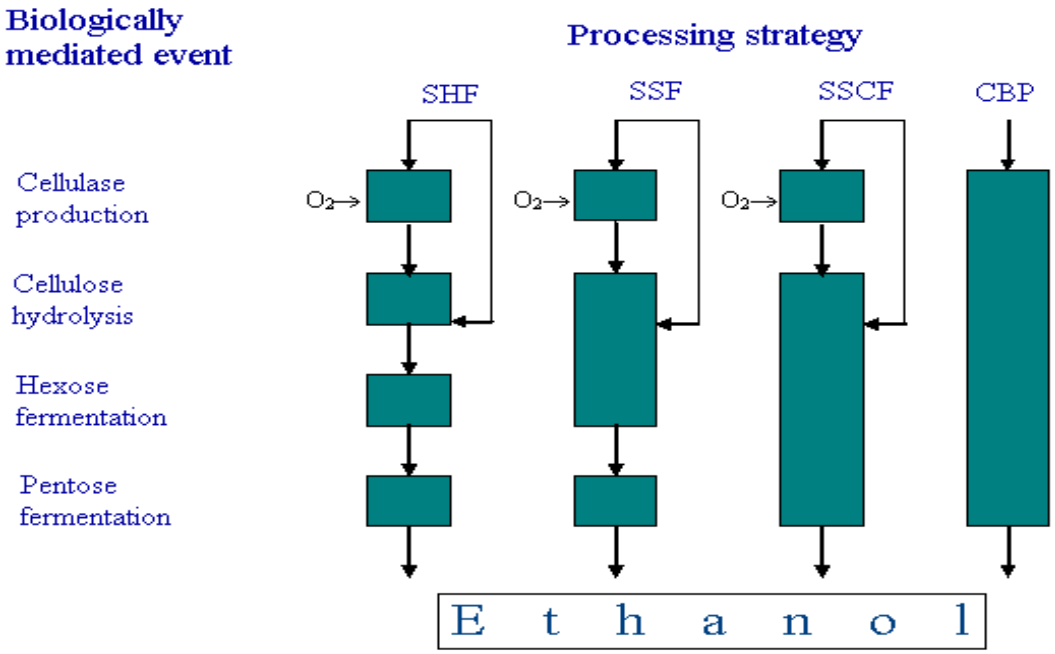


Source: (Shoham *et al*., 1999)

Figure 1.7: Simplified Schematic View of the Interaction Between the Cellulosome and its Substrate, as well as its connection to the Surface via an Anchoring Protein.

Anaerobes depend solely on glycolysis for cellular energy, which means that they are unable to produce an abundance of extracellular proteins. For this reason, the cellulase complex synthesised by *Clostridium thermocellum* has a 50 times higher specific activity than *Trichoderma reesi* cellulases, in the assay conditions used. *T. reesi* is aerobic and produces large amounts of cellulase with rather weak activity (Demain *et al*., 2005).

Anaerobic thermophiles play a minor role in cellulose decomposition in most natural environments because they require unusually high temperatures for growth and cellulose fermentation. Most cellulose is degraded aerobically, but 5-10 % is degraded anaerobically. Anaerobic bacteria can be defined as organisms that function and generate energy without requiring oxygen, and whose oxygen sensitivity renders them unable to grow in the presence of air (Leschine, 1995). Obligatory anaerobic bacteria possess unique metabolic features that provide the perfect conditions for large-scale fermentation (Leschine, 1995).



*Each box represents a bioreactor (not to scale).
SHF: Separate hydrolysis and fermentation
SSF: Simultaneous saccharification and fermentation
SSCF: Simultaneous saccharification and co-fermentation
CBP: Consolidated bioprocessing

(Source: Lynd, 1996)

Figure 1.8: Evolution of Biomass Processing Schemes Featuring Enzymatic Hydrolysis

The thermophilic bacteria offer many benefits. Thermophilic bacteria have subtle structural differences in proteins, nucleic acids and lipids. As a result of growth at high temperature and unique thermally stable macromolecular properties, thermophilic bacteria possess a high metabolic rate, physically and chemically stable enzymes, and lower growth but higher product yields as opposed to similar

Table 1.8: Average Cost Estimations for the Production of Fuel Ethanol by the Fermentation in Industrial Scale Plants (data of different studies)

Feedstock	Europe			USA		
					Lignocellulosic Feedstock	
	Wheat	Rye	Sugar beet	Corn	Near-term Poplar wood (NREL 1999)	Start up 2010 Corn Stover (NREL 2002)
Process energy supply	Gas/ coal	Gas/ coal	Gas/ coal	Gas/ coal	Residue (lignin)	Residue (lignin)
Ethanol production capacity (million litres/year)	> 50	60-260	> 50	> 50	198	262
Ethanol yield (litres/mg if feedstock)	345-385	383	100	370-470	283	318
Feedstock costs/litre ethanol	0.24-0.34€	0.24-0.26 €	0.20-0.44 €	0.22 $	0.097 $	0.088 $
Operating costs/litre ethanol	0.19-0.27 €	0.25-0.31 €	0.20-0.24 €	0.17 $	0.282 $	0.219 $
Co-products benefit/litre ethanol	0.06 €	0.25-0.31 €	0.03-0.07 €	0.09 $	0.019 $	0.025 $
Total production costs /litre ethanol	0/41-0.60 €	0.49-0.55 €	0.04-0.62 €	0.30 $	0.36 $	0.282 $
Total production costs per litre gasoline equivalent	0.62-0.91€	0.74-0.83 €	0.64-0.94 €	0.46 $	0.55 $	0.43 $

Source: (Schieder, 2005)

Table 1.9: Examples of Lignocellulose Degrading Microbial Consortia Isolated from a Variety of Sources as Indicated.

Consortia isolation and composition	Substrate	Major conclusions	Reference
Enriched from soil (*Ralstonia* sp., *Clostridia* sp., uncultured *Firmicutes, Propionibacterium acnes*, uncultured *Betaproteobacterium, Pantoea* sp.*)*	Raw corn stover powder (RCSP) and filter paper	Degraded 51% RCSP and 81% of filter paper in 8 days under facultative anoxic conditions at 40ºC with acetate as the primary product	Feng *et al.* (2011)
Enriched from a variety of lignocellulosic substrates (*Pseudomonas byssovorax, Microbium oxydans, Bacillus Sp., Ochrobactum anthropic*, and *Klebsiella trevisanii)*	Bermuda grass	Characterised enzyme activity of consortia demonstrating a diverse array of cellulolytic and xylanolytic enzymes	Okeke and Lu (2011)
Enriched from cellulose faeces mixtures (*Clostridium* sp., uncultured *Betaproteobacterium, Brevibacillus* sp and *Pseudomonas* sp.)	Filter paper, printing paper, cotton rice straw	Stable community degraded 60% of rice straw, 88% of cotton and 79% of filter paper within four days at 50ºC with ethanol as a significant product	Haruta *et al.* (2002)
Isolated from mature compost windrow (9 species isolated	Filter paper, cotton and rice straw	Degraded 99% of filter paper, 77% of cotton and 81% of rice straw, after 3 days at 50ºC without considering product formation	Wang *et al.* (2011)
Isolated from compost, dung and soil (unknown composition)	Sugarcane bagasse (SCB) and filter paper	Degradation of 77% of alkali-treated SCB in 6 days and 85% of filter paper in 4 days at 50ºC while displaying substrate-bound cellulases	Lu *et al.* (2008)
Isolated from a variety of lignocellulosic materials (*Bacillus subtilis, Streptomyces* sp., *Cellulomonas* sp.)	Pretreated sugarcane leaves	A mixture of 4 of 9 isolated strains degraded 90% compared to a mix of 60% for a combination of all 9 strains in minimal slats medium	Guevara and Zambrano (2006)
Isolated from high-temperature sugarcane bagasse compost (8 major groups including *Clostridium* sp., *Thermoanarobaterium* sp., *Rhodocyclaceae* sp.)	Bagasse, rice straw and corn stover	Stable community displayed a multi-species enzyme system and degraded up to 70% of rice straw, 70% of corn stover and 60% of bagasse in 7 days at 50ºC	Wongwilaiwalin (2010)

Source: (Schieder, 2005)

mesophilic species. The membrane lipids of extreme thermophiles contain more saturated and straight-chain fatty acids than mesophiles, which allows thermophilic bacteria to grow at higher temperatures by providing the correct degree of fluidity required for membrane function (Zeikus, 1979).

The kinetic properties of thermophilic metabolism and enzymes offer some engineering advantages. Fast metabolic rates coupled with high-temperature activity and stability suggest less expensive temperature control for heating or cooling during industrial-scale fermentations. Thermophilic processes also have the potential for shorter retention times, higher loading rates and increase volume reactors. The promise of lower-cost volatile product (ethanol) recovery at microbiologically generated high temperatures is a significant advantage of using thermophiles. These features are especially important in massive volume processes, such as industrial ethanol production (Zeikus, 1979). Figure 1.8 shows the evolution of biomass processing schemes featuring enzymatic hydrolysis.

Fermentation at high temperatures (60-70^{o}C) eliminates or reduces the need for power-consuming cooling or aeration of large fermenters. High temperatures facilitate constant evaporation and distillation of volatile ethanol, prevents the accumulation of ethanol in the fermenter, avoiding the requirement for ethanol tolerant cultures. Only moderate tolerance is required (Demain, Newcombe and Wu, 2005). Anaerobic thermophiles facilitate a consolidated bioprocessing process strategy (Figure 1.8) and eliminate the inefficiency of separate stages for bioprocessing lignocellulose to ethanol.

1.1.16 Mixed Bacterial Consortia

Using pure cultures for raw lignocellulose bioconversion has met with limited success as there are no single bacteria that can degrade both hexoses and pentoses at the same time to produce ethanol. Hence, direct bioconversion of raw lignocellulosic materials is economically more favourable (Pan *et al*., 2006). Attempts have been made to utilise co-cultures of known organisms based on their metabolic relationships, but this approach cannot fully use the richness of natural

genetic resources because just only 0.1–10% of microorganisms thriving in nature can be cultivated under laboratory conditions. Furthermore, biodegradation of lignocellulosic biomass is much more complex involving synergism among microorganisms in the environment that cannot be entirely reconstituted by simple mixtures of known organisms (Feng *et al*., 2011).

In nature, lignocellulose is degraded cooperatively by many microorganisms (Wang, 2011). A mixed consortium isolated from natural environments, which includes *C. thermocellum* would be more efficient pure single bacterial culture. Consortia are interactive groupings of microorganisms ranging from defined dual-species communities to undefined, multispecies aggregations. Such consortia have demonstrated enhanced characteristics over monoculture approaches in the conversion of cellulose and other sugar mixtures to ethanol (Lynd *et al*., 2002) with each bacterial component providing a different advantage to the survival and function of the whole. (Pan *et al*., 2006).

Consolidated bioprocessing (CBP), shown in Figure 1.8, has been introduced as a single process negating the high cost of a dedicated process step for producing cellulase and xylanase enzymes and directly converts lignocelluloses into ethanol (Lynd et al., 2005). A consortium of bacteria would be able to do both. Lignocellulose-degrading consortia have been isolated, including thermophilic consortia (Wang *et al.*, 2011; Liu *et al.*, 2009). Such communities are difficult to analyse since they may contain microorganisms that are difficult or currently impossible to cultivate.

Enrichment culture is a powerful tool to acquire microbial consortia that are close to those functioning in nature. A microbial consortium might be enriched with the ability to utilise raw lignocellulosic biomass synergistically as in nature (Feng *et al.*, 2011).

1.1.17 Economic Considerations

Climate change is a serious global threat. The benefits of vigorous and early action far outweigh the economic cost of not acting. Climate change will affect the

fundamental elements of life for people around the world – attainability of water, food production, health and the environment. Millions of people would suffer hunger, water shortages and coastal flooding as a consequence of global warming. The Stern Review estimates that if we don't act, the overall costs and risks of climate change will be equivalent to losing at 5-20% of global GDP each year and forever (Stern, 2008).

The investment that takes place in the next 10 to 20 years will have a devastating effect on the climate from the middle of the century. Our actions now and over the coming decades could threaten extensive disruption to economic and social activity, on a scale similar to those associated with the world wars and the economic depression of the first half of the 20th century. And it will be difficult or impossible to undo these changes (Stern, 2008).

Socolow *et al.* (2004) have provided a model stabilisation wedge to achieve a flat trajectory of CO_2 emissions. The model requires that no-carbon and low carbon energy strategies must be implemented on an enormous scale across all sectors of the economy and in countries at all stages of economic development.

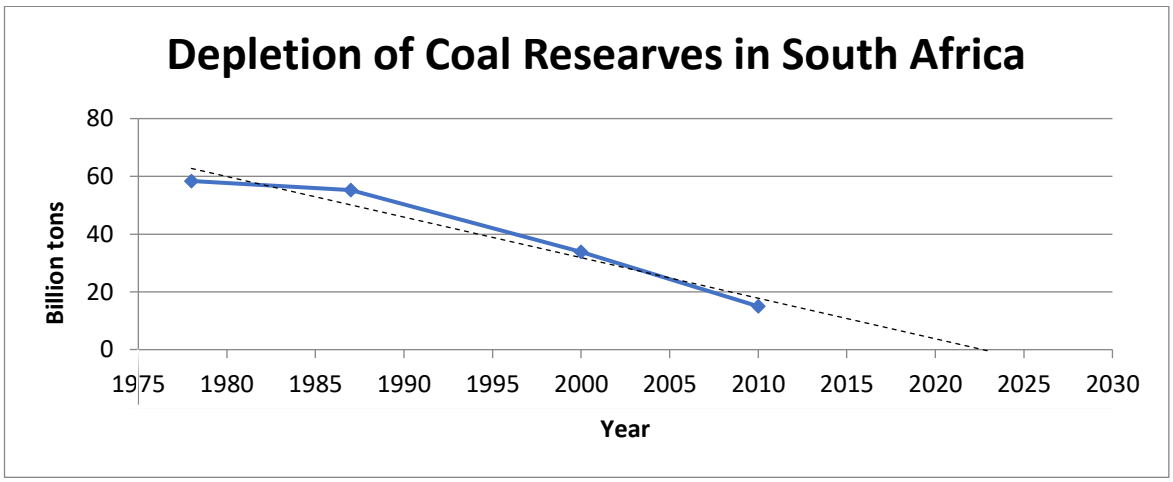
Bioethanol from starch, sugar and cellulose is not yet competitive with conventional liquid fuels. As technology improves, substantial cost reductions will make bio-ethanol a competitive alternative. The cost of *T. reesi* cellulase is still prohibitively high, as is the expense of acid pre-treatment which does not have the potential of cost reductions either (Wyman, 1999)

The direct anaerobic route has the highest potential for cost reduction. Glucose is produced during the fermentation of cellulose to ethanol, eliminating an enzyme cost factor. Production of cellulase, lignocellulose hydrolysis and fermentation take place in an individual bioreactor, reducing the cost of separate processes as is currently required in the case of *T. reesi-Saccharomyces cerevisiae* that operates at different optimal temperatures. Thermophiles have a higher conversion than using *T. reesi* cellulase. Mixed cultures of *C. thermocellum* could reduce costs by half. Using all possible agricultural, forest and urban feedstocks, conversion to ethanol could become economically viable when oil prices are about $30 or more per barrel.

T. reesi would take longer as it is more expensive. Significant advantages include voiding the costs for cellulase production, elimination the need for separate incompatible enzyme and fermentation systems.

1.1.18 The South African Context

South Africa is currently undergoing an energy crisis, as can be seen in the increasing prevalence of 'load-shedding'. Due to its widespread availability, South Africa is dependent on coal for 72% of its energy needs. Crude oil provides 22%, and less than 10% is supplied by nuclear, gas and renewable fuels. This heavy dependence on coal has caused the coal reserves to be depleted much sooner than expected. Following the current trajectory, the coal reserves will be consumed within 5 years, i.e., by 2024 (Sekoai and Daramola, 2017). The depletion of South Africa's primary feedstock for electrical energy production will throw the country into huge problems if no alternative has been found by then.



Source: Sekoai and Daramola, 2017

Figure 1.9: Projected depletion of coal reserves in South Africa

South Africa produced the highest CO_2 levels in Africa in 2011 (40%) and 1.2% of CO_2 globally. The increasing energy demands have drastically increased the levels of CO_2 emissions by 18% from 2001 to 2011. The country's power parastatal Eskom has been under immense pressure because of the county's escalating energy

demands (Sekoai and Daramola, 2017). South Africa has limited oil reserves; therefore, and 70% of the fuel consumed must be imported. It is more important than ever for South Africa to diversify its energy supply (Pradhan and Mbohwa, 2014).

South Africa has massive untapped clean alternative energy resources like biomass, wind, solar, and marine energy that could be used to reduce carbon dioxide emissions and improve the country's energy security. There are 17.3 million tons of agricultural and forestry waste and 8,7 million tons of invasive species available each year that could be used as biofuels feedstocks (Pradhan and Mbohwa, 2014).

Table 1.10: Ethanol plants in the pipeline in South Africa

Name	Type of feedstock	Capacity (million L/year)	Location
Arengo 316P. Ltd.	Ethanol (sorghum)	90	Cradock, Eastern Cape
Mabele Fuels	Ethanol (sorghum)	158	Bothaville, Free State
Ubuhle Renewable Energy	Ethanol (sugarcane)	50	Jozinin, KZN
E10 Petroleum Africa CC	Ethanol	4.2	Gauteng, Germiston

Source: Pradhan and Mbohwa, 2014

In South Africa, the bioethanols industry is still in its infancy even though first-generation bioethanol production is a mature technology and is widely used globally. Second generation cellulosic ethanol still requires a lot of research and development and will follow swiftly after first-generation bioethanol production is established

The South African policy on renewable energy includes biomass energy but is mainly focused on first-generation feedstocks like sugar, maize and sugar beet. Cellulosic crop waste under consideration is sugarcane bagasse and crop waste from maize, wheat, barley and sorghum. The use of sugar, food grains for biofuel production challenges food security, social and economic concerns, commodity prices, and impacts land-use changes on the environment. The biofuels policy aims to contribute 2% biofuels into the national liquid fuel supply (400 million

litres/annum). The biofuels strategy has been in place since 2007, but delays in implementation, political inaction, and food security concerns have hindered the biofuels industry in South Africa (Pradhan and Mbohwa, 2014). At an energy conference in October 2018, the recommitment to this biofuel blending policy to be finalized and approved by the cabinet in early 2019. Despite this, four bioethanol plants are due to be developed, Table 5.2.

1.2 MOTIVATION

Society today is confronted with dwindling sources of fossil fuels and the proliferation of lignocellulosic wastes generated by municipalities, agriculture and industries (Zeikus, 1980). Fossil fuel usage is a chief contributor to greenhouse gases and global warming. The Kyoto protocol declared that all first world countries should decrease their CO_2 output by 10% by 2012. Also, the hike in oil prices in the 1970s, which caused the fuel crisis, resulted in the realisation that the earth's oil supply is not infinite. The pursuit of alternative renewable fuels began in 1975 with the Brazilian ethanol program (from sugarcane) and the USA ethanol program (from corn).

Table 1.11: Benefits and Risks of Biofuels

Advantages	Risks
• Crude oil reserves extended (intergenerational benefit) • Benefits from increased expenditure on fuels to agriculture - Job creation - Farmers - Agribusiness - Rural poor • Sustainable development - Job creation • Reduces pollution • Low net CO_2 release • Co-products	• Poor results for nitrous oxide emissions • Competition for land with food and fibre production • Transportation of large volumes of low energy density biomass fuels • Impacts of processing (stillage) • Hedge against high fuel prices • Water requirements

The potential amount of ethanol that could be produced from cellulose is over an order of magnitude larger than that obtainable from starch or sugar-based feedstocks. As a result, cellulose-degrading microorganisms that have gained interest in recent years (Demain *et al*., 2005).

Several factors make mixed consortia perfectly suited to ethanol production from lignocellulosic feedstocks. Firstly, *C. thermocellum* in the consortium can utilise lignocellulosic waste and generate ethanol. Anaerobic fermentation is preferable for large-scale fermentation because one of the costliest steps in industrial-scale fermentation is that of supplying adequate oxygen transfer as is required to manufacture cellulase. The high-temperature culture conditions reduce the problems of contamination, dramatically simplifies the cooling of large fermenters, and facilitates the recovery of ethanol. Robust thermophiles produce temperature-stable enzymes. The low cell yield of anaerobes promotes substrate conversion to ethanol. In situ cellulase production and the high rates of growth and metabolism of cellulose and hemicellulose are very advantageous. Since the growth of plants recycles carbon dioxide produced during fermentation and fuel use of ethanol, ethanol does not contribute to CO_2 accumulation in the atmosphere and possibly global warming.

The focus of this study is threefold: the isolation and screening of thermophilic, anaerobic spore formers that degrade cellulose produce ethanol at a high rate of production from cellulose; testing the efficiency of the isolated bacterial consortium in an AFBR; characterization of the bacterial consortium using DGGE.

CHAPTER 2
SELECTION OF BACTERIAL CONSORTIA

2.1 INTRODUCTION

The obligate cellulolytic anaerobic bacterium *Clostridium thermocellum* was initially chosen as the model organism because it is an ethanologenic thermoanaerobe with substrate attachment properties due to its cellulosome. The routine culture of *Clostridium thermocellum* is dependent on the anaerobic hood and the strict application of Hungate procedures (Hungate, 1944). Also, *Clostridium thermocellum* requires complex media, including yeast extract and various vitamin supplementations (Johnson *et al*., 1981). The need for a costly complex medium as well as dependence on strict anaerobic conditions was circumvented by selecting for an undefined consortium of cellulolytic mixed bacterial species instead of a single, strict anaerobe, i.e., *C. thermocellum*. The consortia would include both facultative anaerobic and obligate anaerobic bacterial species (Qian *et al.*, 2011). Due to syntrophic interactions and metabolic cooperation, the multispecies bacterial consortia would be able to grow on simpler nutrient media formulations, especially favourable when running a large-scale bioreactor. The presence of facultative anaerobes would allow for the adoption of "Non-Hungate" culturing procedures, and the dependence on the anaerobic hood would be negated.

2.1.1 Anaerobic Spore Forming Bacteria

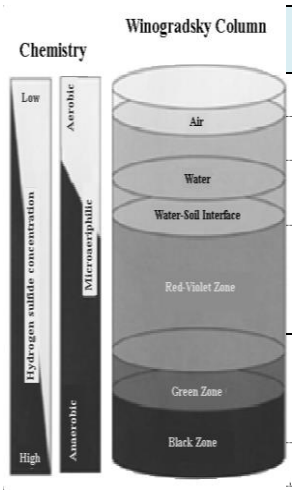
Endospores are formed under various experimental conditions by members of many genera of bacteria, such as Bacillus and Clostridium, which are widely distributed, especially in soil. An essential and advantageous aspect of the bacterial endospore is the degree of both heat resistance and survival in nature (McCaul and Williams, 1981). The environmental samples would be obtained under aerobic conditions, but the heat treatment required for endospore selection eliminates all vegetative other non-spore forming bacteria.

2.1.2 Winogradsky Column Enrichment Culture

The Winogradsky Column is a simple tool for culturing a broad diversity of microorganisms. It was developed and named after Sergei Winogradsky (1856-1953), a Russian microbiologist. Most microbiologists of the time, such as Louis Pasteur and Robert Koch isolated pure cultures for study, but Winogradsky's was the first to study mixed environments of microorganisms. He studied the complex interactions between environmental conditions and microbial activities using soil enrichment to study microbes in diverse enrichment cultures (Chung and Bryant, 1997). The apparatus is a column of pond mud and water mixed with a cellulose-containing carbon source such as newspaper or egg-shells (containing calcium carbonate) and a sulfur source such as gypsum (calcium sulfate) or egg-yolk. Incubating the column in a sunny or shady environment for a few months results in an aerobic-anaerobic gradient as well as a sulfide gradient (Rogan *et al*., 2005).

The Winogradsky column spatially separates organisms into distinct layers, several centimetres thick. It establishes conditions that expand the volume of natural processes, allowing a clear view of naturally-occurring phenomena. Soil samples are collected from wetland habitats, supplemented with simple inorganic and organic materials and then exposed to light as an external energy source or kept in the shade (Chung and Bryant, 1997; Demain *et al*., 2005)).

The result is a multicoloured column of soil and water, illustrated in Figure 2.1, with each layer linked to a different chemical or biological process. The defined zones of microbes develop according to the concentration gradients of oxygen, sulfur, nutrients, and light. Aerobic bacteria will be concentrated at the aerobic end (top surface), and anaerobic bacteria will be at the anaerobic end (bottom) of the concentration gradient. Each functional microorganism group is dependent on other functional microbial groups for development (Rogan *et al.,* 2005). The anaerobic end of the Winogradsky column provides an excellent mixed enrichment culture from which to isolate an anaerobic cellulose-degrading consortium. The Winogradsky column has been confirmed to be useful as a more specific methodology for the enrichment of environmental samples for hydrogen and ethanol-producing bacteria (Loss *et al*., 2013).



Organisms	Representative genus	Morphology	Gram stain	Metabolism
Diatoms	Various	Silica frustules	N/A	Photosynthetic
Cyanobacteria	Various	Singular or filament	N/A	Photosynthetic
Protists	Various	Singular or simple colonies	N/A	Photosynthetic or Heterotrophic
Sulfur-oxidising bacteria	- Beggiatoa	Filamentous	Negative	Non-photosynthetic Chemolithotrophic
	- Thiobacillus	Rods	Negative	Chemolithotrophic
Purple non sulfur bacteria	- Rhodospirillum	Vibrio spiral	Negative	Chemolithotropic
	- Rhodopseudomonas	Rods	Negative	Photoheterotrophic
Purple sulfur bacteria	Chromatium	Ovals or rods	Negative	Photoheterotrophic
Green sulfur bacteria	Chlorobium	Straight or curved rods	Negative	Photoheterotrophic
Sulfur reducing bacteria	Desulfovibrio	Vibrio	Negative	Chemoheterotrophic
Non-sulfur obligate anaerobic bacteria	Clostridium	Rods with endospores	Positive	Fermentative

Source: (Rogan *et al.*, 2005)

Figure 2.1: Typical Bacterial Gradient Occurring in the Winogradsky Column. Bacterial species grow in distinct layers determined by the oxygen and hydrogen sulfide concentration gradients that form between the top to the bottom of the column.

2.1.3 Aims

The focus was the isolation and selection of a mesophilic (37°C), and thermophilic (65°C) anaerobic cellulolytic bacterial consortium from a few sources that degrade lignocellulose efficiently produces ethanol.

2.1.4 Objectives

Collect environmental samples and culture Winogradsky columns as an enrichment culture to provide environmental samples for inoculation. Use anaerobic hood, Hungate, and microaerophilic culture methods to inoculate environmental samples into serum vials containing medium containing cellulosic substrate as sole carbon source. Cultivate 37°C and 65°C to identify the mesophilic and thermophilic cultures that degrade filter paper fastest. Consolidate the most efficient cultures to form final mesophilic and thermophilic consortia.

2.2 MATERIALS AND METHODS

2.2.1 Presumptive Test for the Presence of Thermophilic Anaerobes using an Anaerobic Hood

This presumptive test was performed to test the success of strict anaerobic culture techniques using the Anaerobic Hood. A commercially produced anaerobic medium customarily used to test for the presence of pathogenic Clostridia, Differential Reinforced Clostridial Medium (DRCM) (Appendix A), was used to determine if thermophilic anaerobes could be cultured using a general Clostridium medium. The presence of sulfate-reducing Clostridia, which are faecal indicators and are commonly used to evaluate sanitary conditions (Robles *et al.*, 2000), is detected using DRCM detects. Growth is indicated by the blackening of the culture medium caused by the addition of sodium bisulfite and ferric citrate. The aim of this pilot study served as a trial to confirm the success of the anaerobic hood to create anaerobic culture conditions.

Ordinary garden soil was used as an environmental sample for this trial. The following oxygen-sensitive steps were carried out in an Anaerobic Hood utilising a mixture of anaerobic gases (5% H_2 gas mix):

1. The media was made up as per instructions on the bottle and dispensed (20ml) into 24 serum vials (120 ml) (Wheaton).
2. Serum vials were then autoclaved for 20 minutes at 121 psi.
3. The autoclaved serum vials were immediately transferred directly into an anaerobic hood to prevent oxygen from re-dissolving into the media while cooling down.
4. Soil samples (2g) were inoculated into serum vials containing medium, after which the serum vials (Merck) were sealed. Sterile Butyl rubber stoppers (Merck) and aluminium lids (Merck) were used to seal the serum vials with the aid of a crimper (Wheaton). A heat killing step to kill all vegetative bacteria is usually indicated but was not performed.

5. The serum vials were then removed from the anaerobic hood and incubated at 70°C at 120 rpm for 15 days.

A heat killing step to kill all vegetative bacteria is indicated but was deemed unnecessary for this presumptive test. It was unimportant whether a positive result was obtained from spores or vegetative bacteria. A positive test would show that thermophilic bacteria could be grown in anaerobic conditions using the Anaerobic Hood for anaerobic manipulations.

2.2.2 Selection of Cellulolytic Anaerobes using the Hungate Method of Anaerobic Manipulation

The use of the Anaerobic Hood would have been ideal for strict anaerobic culture, required by strict anaerobes such as *C. thermocellum*, but a constant, reliable supply of the anaerobic mix of gases needed could not be guaranteed at the time. An alternative to the use of an Anaerobic Hood for maintaining anaerobic culturing conditions is the Hungate method, which entails the continuous gassing of serum vials with an anaerobic gas during manipulation.

(a) Sample Collection

The soil was collected from various sites, Table 2.1, which are likely to contain cellulolytic spores, to test the success of strict anaerobic culture techniques using the Hungate method. After collection, soil and water samples were put into plastic bags/bottles and stored in the fridge at - 4ºC before being used.

(b) Inoculation

1. Minimal medium (Appendix A) containing resazurin indicator without reducing agent was dispensed (20ml) into serum vials containing 30mm diameter Whatman 1 filter paper disks and autoclaved for 20 minutes.
2. After cooling, each serum vial was inoculated with 2g of soil or 2ml of the liquid sample and gassed for five minutes using ultra-high purity (UHP)

nitrogen gas to exclude oxygen, then sealed with a butyl rubber stopper and aluminium lid.

3. The reducing agent, L-Cysteine, was added after the serum vials were sealed, through the butyl rubber stopper using a 1ml plastic syringe.
4. A heat kill step was performed at 95°C for 20 minutes in a water bath to eliminate all vegetative growth, sulfate-reducing bacteria and methanogens (Ghimire *et al.*, 2015).
5. Sealed vials were then incubated at 65°C for 20 days without agitation.

The indicator used, resazurin, is purple before being reduced pink (pre-reduced) after autoclaving and clear (reduced) after the addition of reducing agent (L-Cysteine). In this way, the medium was anaerobic before inoculation and remained so afterwards.

(c) Subculture

1. Serum vials were prepared in the same way as they were for inoculation.
2. Duplicate serum vials were inoculated with the culture that displayed growth with filter paper degradation. 2ml of culture was removed by syringe through the butyl rubber stopper and inoculated into fresh anaerobic medium prepared as previously described.
3. Sealed vials were then incubated at 65°C for a further 20 days.

Table 2.1: Inoculum Sources for Thermophilic Anaerobic Culture using the Hungate Technique

1. Old Nematode Soil	11. Dung Beetle Fecal Deposit
2. Compost 1	12. Fresh Dung
3. Compost 11	13. Dung Beetle Brood Balls
4. Leaf Compost	14. Dung used by Dung Beetles
5. Worm from leaf Compost	15. Dung beetle Brood Balls (Fecal Deposit)
6. Cow Dung	16. Lawn Mulch
7. Rotting Lawn Mulch	17. Termite Mound Soil
8. Swamp Soil	18. Field Ant Soil
9. Swamp Water	19. Water sample Warm Baths Hot Spring
10. Used Dung Beetle Soil	20. Sewage

2.2.3 Selection of Cellulose Degrading Bacterial Consortia using Microaerophilic (Non-Hungate) Culture Technique

Cellulolytic *Clostridia* are spore formers, which do not require anaerobic conditions until after inoculation and vegetative growth has begun. The selection of a consortium containing both facultatively anaerobic and obligate anaerobes bypasses the problems associated with strict anaerobic culture requirements (Loss *et al*., 2013). The aerobic spore formers would remove all oxygen from the medium, which provided suitable conditions for the anaerobic spore formers to take over.

(a) Assembly of Winogradsky Enrichment Cultures

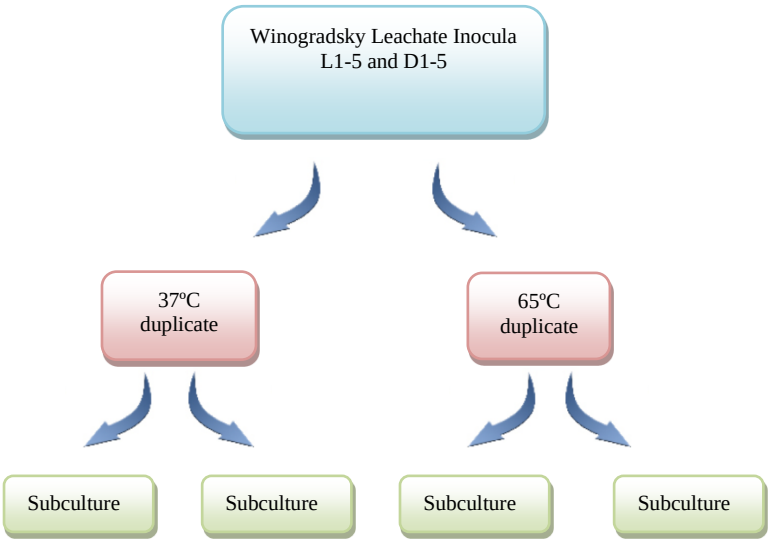
Winogradsky Columns were constructed and used as enrichment cultures. An enrichment culture was the best option to increase the amount of anaerobic cellulolytic bacteria in our environmental samples.

Table 2.2: Sources of Inoculum used in Winogradsky Columns

.1. Pond water and mud was collected from a pond on the campus of the University of the Witwatersrand.
2. Pond 2 water and mud were collected from another pond on campus.
3. Swamp water and mud was collected from a swamp close to the University.
4. Lake water and mud was collected from a lake close to the University.
5. Pond water and worm compost from home worm composter.

A two-litre plastic cold drink bottle with the top cut off was used as a culture vessel for the columns. One litre of mud was mixed with some newspaper and supplemented with 5g calcium sulfate, 1g magnesium sulfate, and 2.5g calcium carbonate were mixed used to fill $^{2}/_{3}$ of the 2-litre bottle. Water was then added to saturate the mud and occupy half the remaining volume. The top of the column was sealed with parafilm to prevent

evaporation of the water. Five different columns were prepared in duplicate, using water and mud samples collected from the following sites:



And so on…

Figure 2.2: Winogradsky, Round 1, Subculture Procedure.

(b) Inoculation

The water and soil from the Winogradsky columns were thoroughly mixed, and liquid leachate was collected in a 100 ml Schott bottle, shown in figure 2.2 (e), and used for inoculation of serum vials. 1 ml of culture was removed from serum vial using a sterile plastic syringe and inoculated into serum vial with the fresh sterile aerobic medium. Each selected culture was sub-cultured in duplicate, as illustrated in Figure 2.2.

Approximately 2ml of Winogradsky leachate was inoculated into serum vials prepared with 20 ml ATCC 1190 medium without reducing agent, 30mm Whatman 1 filter paper disks, and 40mg of sawdust made from hamster wood shavings, shown in Figure 2.4 (c) and (d). No reducing agent was added to the pre-reduced medium

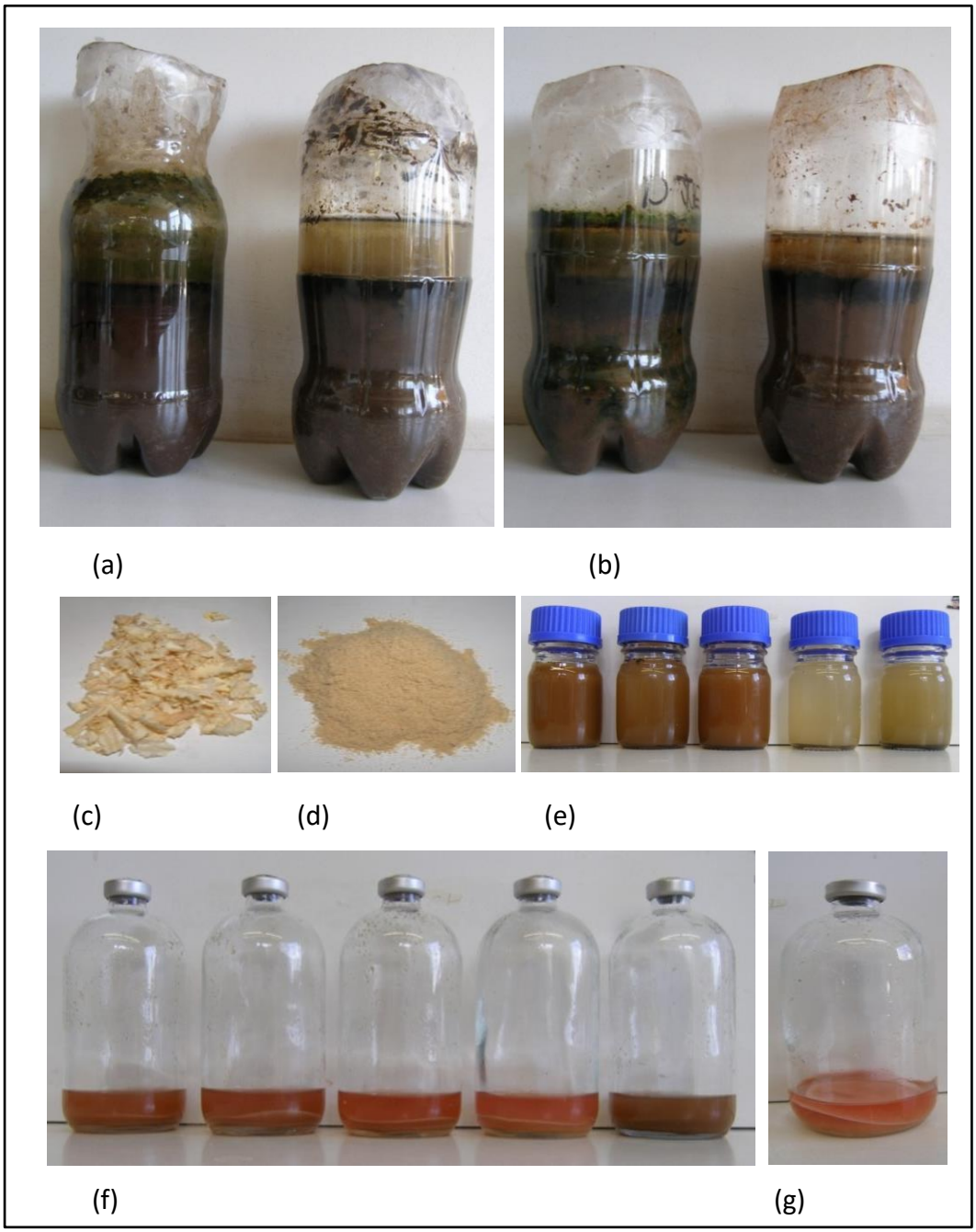


Figure 2.3: Inoculation of Serum Vials. Duplicate Winogradsky columns, with one incubated in the light (a) and one in the dark (b). The different layers of microorganism growth can be seen. (c) Wood shavings (hamster bedding) before milling and (d) sawdust formed after milling of the wood shavings. (e) Leachate samples collected from the

Each leachate sample was used to inoculate two sets of duplicate serum vials (120 ml). One set was incubated at 37°C and one at 65°C without agitation. Figure 2.4

(f) shows how the inoculated serum vials looked, with (g) being the uninoculated control.

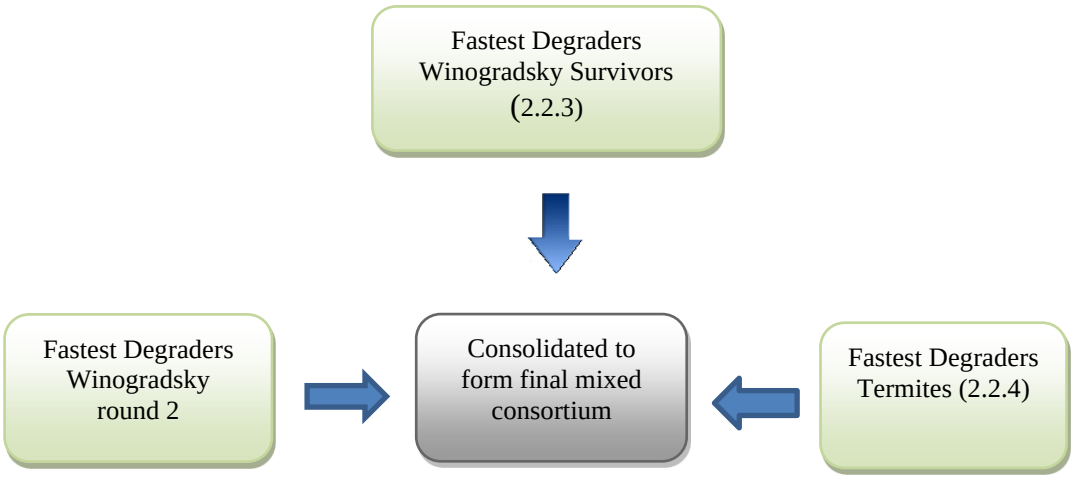


Figure 2.4: Procedure used for the selection of cultures for consolidation to form the final 37°C and 65°C mixed Consortia.

(c) Subculture

Serum vials were removed from the incubator when the filter paper was degraded entirely and left on the benchtop for at least one day before being sub-cultured to encourage spore formation that would enable microaerophilic subculture that otherwise would immediately kill the vegetative cells of all cellulose-degrading anaerobes.

2.2.4 Construction of Consolidated Cultures for Bioreactor Inoculum

A three-pronged approach was followed, figure 2.4b, to obtain a cellulose-degrading consortium with the highest diversity of facultatively anaerobic and obligate anaerobic, microaerophilic consortium, with the highest chance of forming the most competitive and stable microaerophilic inoculum for the following bioreactor phase (Chapter 3),

Winogradsky columns, used as inoculum for serum vials. (f) Serum vials inoculated with leachate from the five different columns. (g) Uninoculated control.

Firstly, the Winogradsky leachate samples (D1-5 and L1-5) were re-inoculated, and the first degraders, instead of the full degraders were sub-cultured. The cultures that started degrading fastest was selected to be incorporated into the final consortium. Secondly, the best degraders form the first round of Winogradsky cultures were sub-cultured, after an extended period of dormancy in the cold room (-4°C) to ensure their viability before selection for the final mixed consortium. Thirdly, consortia were selected from termite inoculations to be incorporated into the final consolidated consortia. 5ml of each selected mesophilic and thermophilic cultures were consolidated to form the final mixed consortia.

(a) Selection of Cellulose Degrading Bacterial Consortia from Second Round of Winogradsky Leachate Inoculation

The Winogradsky leachate samples (L1-5 and D1-5) were again inoculated into serum vials with ATCC medium, filter paper disks and 40mg of wheat bran. A triplicate of each leachate sample was incubated at 37°C and a triplicate at 65°C. The first of each leachate sample to display filter paper degradation was removed from the incubator for subculture. The first of the sub-cultures to show filter paper degradation were selected to form part of the final consolidated consortium.

(b) Selection of Best Cellulose Degrading Bacterial Consortia from the First Round of Winogradsky Inoculations

Cultures that degraded cellulose best from the first round of Winogradsky inoculations were sub-cultured into fresh medium and incubated after remaining dormant in the fridge for an extended period to test for viability before selection for the final mesophilic and thermophilic mixed consortia.

(c) Selection of Cellulose Degrading Bacterial Consortia from Termites

Sample Collection

Termites were collected from a termite mound at the Melville Koppies Nature Reserve in Johannesburg. After collection, termites were placed in a plastic bag and stored in the fridge at -4°C.

Inoculation

1. Five termites per serum vial were macerated using a mortar and pestle were before being used for inoculation.
2. ATCC medium containing resazurin indicator, 30mm Whatman 1 filter paper, and 40g of hamster shaving sawdust without reducing agent was prepared and dispensed (20ml) into serum vials and autoclaved for 20 minutes.
3. After cooling, each serum vial was inoculated with five macerated termites.
4. A heat kill step was performed at 95°C for 20 minutes in a water bath to eliminate all vegetative bacteria.
5. Five serum vials were incubated each at 37°C and 65°C.

Subculture

Termite cultures that showed the fastest filter paper degradation was selected for subculture. The selected culture was injected anaerobically using a syringe directly into five sealed serum vials. The serum vials were incubated until filter paper degradation was observed. The first of the sub-cultured cultures to show filter paper degradation were selected to form part of the final consolidated consortium.

2.2.5 Measurement of Ethanol Production

The ethanol content of a few cultures from the first round of Winogradsky leachate inoculations (Section 2.2.3), were measured. The cultures selected were the most efficient mesophilic and thermophilic ethanol degraders.

2.2.6 Scanning Electron Microscopy

The thermophilic culture that degraded filter paper the fasted D2i was selected to view under the scanning electron microscope. The following procedure was followed:

All procedures with glutaraldehyde were performed in a fume hood with gloves on. Liquid samples (500µl) were fixed in 3% glutaraldehyde (500µl) overnight. All procedures using glutaraldehyde were performed in a fume hood. Liquid samples were passed through a 2µl filter using a 1ml plastic syringe, but solid samples such as filter paper did not require filtration. Samples underwent an ethanol dehydration series: 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% for 10 minutes each (Lindsay and von Holy, 1997). Samples were kept in 100% ethanol until they were mounted and coated with gold-palladium splutter. The samples were viewed using a JEOL JSM-840 SEM (Japan) at 20Kv and a working distance of 15 mm.

Table 2.3: Anaerobic Culture of Thermophiles using an Anaerobic Hood

# days After Inoculation	Positive Growth (Blackening of Medium)
6	0
7	4
10	5
12	6
13	7
14	8
15	No further change
Total cultures showing positive growth	8/24

2.3 RESULTS AND DISCUSSION

2.3.1 Presumptive Test for the Presence of Thermophilic Anaerobic Using an Anaerobic Hood

The test for anaerobic growth of thermophiles was positive (Table 2.3), with a 33% incidence of thermophilic anaerobes in the soil sample. Differential reinforced clostridia medium is usually used to tests for the presence of pathogenic Clostridia at mesophilic temperatures and not for cellulolytic Clostridia at thermophilic temperatures. The trial, therefore, also showed that the medium supported the growth of bacteria that grow in thermophilic conditions (70°C). The capacity of the reducing agent (L-Cysteine) in DRCM to remove all oxygen from the serum vials, shown by the blackening of the medium, indicated that an anaerobic environment was successfully attained. It also confirmed that the serum vials used for culture maintained an anaerobic environment.

Despite the low incidence of growth, the results confirmed the successful use of the Anaerobic Hood in maintaining strict anaerobic conditions.

2.3.2 Selection of Cellulolytic Anaerobes using the Hungate Method of Anaerobic Manipulation

There was a 35% incidence of growth (Table 2.4). After subculture, no growth occurred at all. A reason for this may be that the reducing agent used was later found to be expired. The expired reducing agent was thought to be responsible for inhibiting the growth of cellulolytic bacteria. Even though the indicator became clear, confirming that the reducing agent removed all oxygen as the expired chemical may have produced the side effect of inhibiting bacterial growth. It was concluded, therefore, that the Hungate technique itself was successful because the sealed culture remained anaerobic.

Table 2.4: Anaerobic Culture of Thermophiles using Hungate Technique

Inoculum Source	20 Days	40 Days
1. Old Nematode Soil	-	-
2. Compost 1	+	+
3. Compost 11	+	+
4. Leaf Compost	-	-
5. Worm from leaf Compost	-	-
6. Cow Dung	-	-
7. Rotting Lawn Mulch	+	+
8. Swamp Soil	-	-
9. Swamp Water	-	-
10. Used Dung Beetle Soil	-	-
11. Dung Beetle Fecal Deposit	-	-
12. Fresh Dung (before dung beetle added)	+	+
13. Dung Beetle Brood Balls	+	+
14. Dung used by Dung Beetles	+	+
15. Dung beetle Brood Balls without Fecal Deposit	-	-
16. Lawn Mulch	+	+
17. Termite Mound Soil	-	-
18. Field Ant Soil	-	-
19. Field Ant Soil 11	-	-
20. Sewage	-	-
	7/20	7/20

2.3.3 Selection of facultative and anaerobic cellulose-degrading bacterial consortia using a microaerophilic culture technique

(a) Selection of mesophilic cellulose degraders

Ten Winogradsky leachate samples were inoculated. Complete degradation of filter paper occurred in three of the Winogradsky cultures, i.e., D_1, D_2 and L_5, shown in Table 2.5. Cultures with no degradation, incomplete degradation, and degradation that took too long were eliminated (Table 2.6).

Culture D_3 was eliminated because one culture did not degrade the filter paper completely, and the other had no degradation. The one D_1 culture inoculation had no filter paper degradation, but the other degraded filter paper entirely after 29 days,

but after sub-culture, it did not degrade filter paper completely and was thus eliminated. Culture L_5 also had incomplete filter paper degradation after subculture. Figure 2.5 shows undegraded filter paper (b) and (c) I, incompletely degraded filter paper (c), and fully degraded filter paper (d), (f), (g), and (e) ii, iii and iv.

Table 2.5: First Round of Isolation (37°C)

# Days for Total Degradation						
Sample	Inoculation	Subculture 1	Sub 2	Sub 3	Sub 4	Average
D_1	29	I				29
D_2	29	24	26	26	I	26
	29	24	I			27
	28	26	28	27	I	27
	28	26	I			27
L_5	22	I				22
	5	I				5
Shortest time	5	24	26	26		
Average time	21	25	27	27		

Key	
I	Incomplete filter paper degradation
N	No filter paper degradation- discarded
	Discontinued culture

Table 2.6: Failed Cultures for First Round of Isolation (37°C)

Cultures	Discarded due to
L_1, L_2, D_{1ii}, D_{3i}	I – incomplete filter paper degradation
L_3, D_{3ii}, L_4, D_4, D_5	N – no filter paper degradation

Culture D_2 was the only one that retained its cellulolytic ability up to the third subculture. After the fourth subculture, however, D_2 also stopped degrading filter paper completely. After inoculation of leachate L_5 broke down the filter paper fastest (5 and 22 days) but did not continue to degrade filter paper entirely after subculture. The viable cellulose-degrading bacteria may have been lost during subculture. It could have occurred because the facultative anaerobes required to remove the remaining oxygen from the pre-reduced medium were lost, preventing the growth of obligate cellulosic bacteria. The cellulolytic capacity of D_2 was retained for longer, which indicated that it was microaerophilic up until the fourth

subculture, when the loss of aerobic bacteria may have been responsible for the loss of degrading ability. The consortium isolated from D_2 was thus found to be the best mesophilic microaerophilic cellulose degrader. Cultures D_1, D_2 and L_5, were selected to perform the second round of leachate inoculations from which the final mixed consortium was selected.

(b) Selection of Best Thermophilic Cellulose Degraders

Five out of the ten leachate samples showed sufficient filter paper degradation (Table 2.7). After subculture, D_3 took 48 days for complete degradation and was discontinued. Cultures L_1, L_2, L_4 and D_4 had no filter paper degradation, and D_5 took more than 35 days for complete degradation and were thus stopped. Culture D_1 retained its degrading efficiency until after the fourth subculture, but others either took too long or incompletely break down filter paper. The fastest degradation took place in one of the D_2 inoculations, i.e., five days. Unfortunately, this rapid rate of degradation was not retained after it was subcultured.

Except for D_1, the cultures all took longer to degrade after subculture. On average, cultures took longer to degrade after sub-culture than after inoculation. After inoculation, cultures took on average 14 days for full degradation, and after sub-culture, it took 24, 22 and 23 days for complete degradation. D_{2ii} degraded filter paper the fastest, i.e., after five days after inoculation compared to the average of 15 days. D_2 was found to be the best degrader, but all five cultures: D_1, D_2, L_3, D_3 and L_5 were selected for the second round of Winogradsky inoculations

Cultures incubated at 65°C degraded filter paper faster than their mesophilic counterparts. After thermophilic inoculation cultures that degraded well, did so in an average of 14 days, compared to the average of 21 days for mesophilic consortia. This culture stopped sufficiently degrading filter paper after the up until the third subculture. D_{1i} and L_{3i} maintained the cellulosic capacity one subculture longer. The cultures that were eliminated before subculture are shown (Table 2.8). Compared to the mesophilic mixed consortium (Table 2.6), the thermophilic cultures (Table 2.7) degraded filter paper faster. On average, the fastest

thermophilic degradation took 15 days long, compared to the fasted average mesophilic time of 21 days.

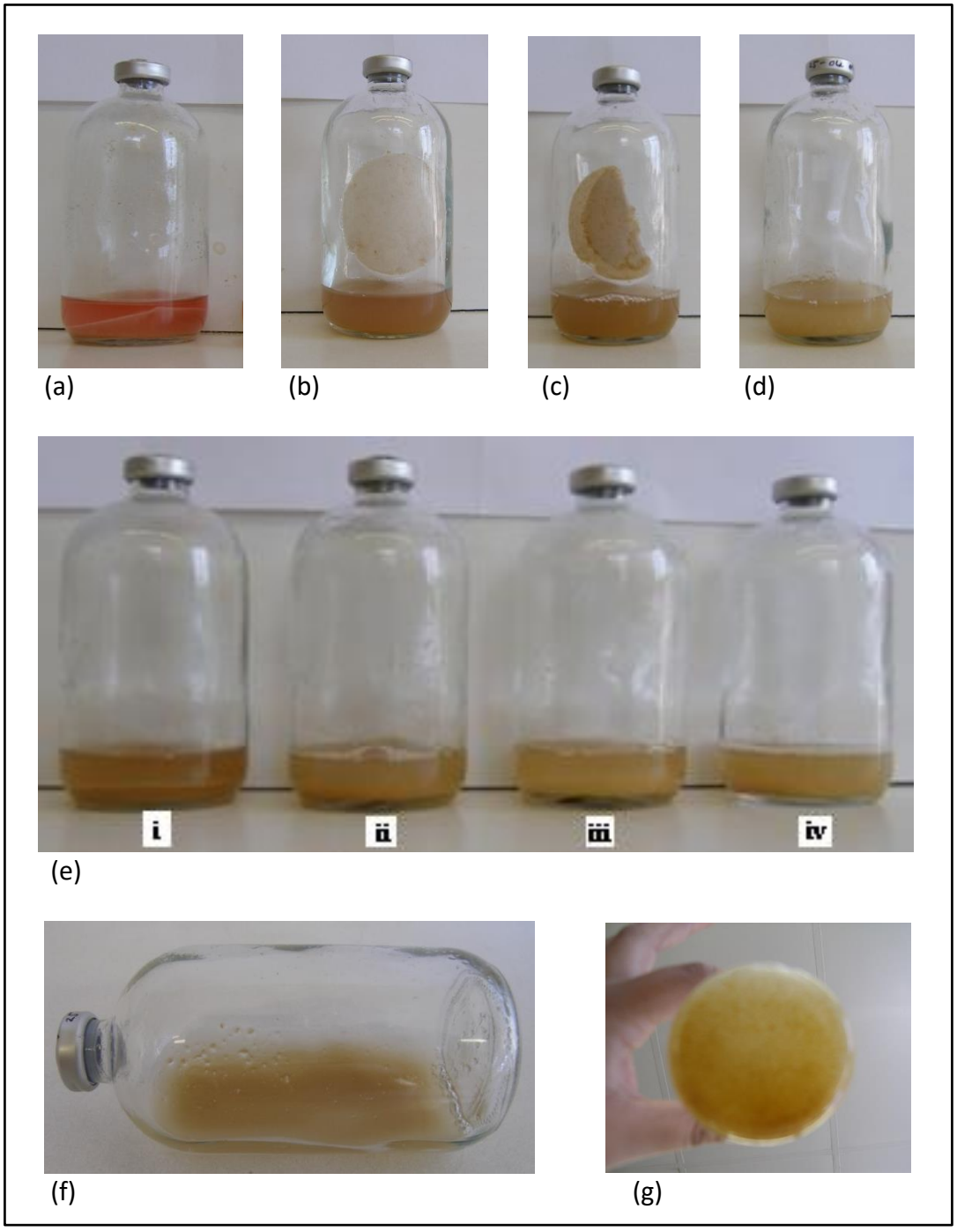


Figure 2.5: Degradation of Filter Paper from Winogradsky Leachate Inoculum

(a) Uninoculated Control (b) and (e) i. Show no filter paper degradation (c) The start of filter paper degradation (d), (f), (g) and (e) ii, iii, and iv Show complete filter paper degradation

Table 2.7: First Round of Isolation (65°C)

# Days for Total Degradation						
Sample	Inoculation	Subculture 1	Sub 2	Sub 3	Sub 4	Average
D_1	28	12	19	13	15	17
					20	18
					27	20
	28	12	19	19	I	20
D_2	15	20	11	I		15
				I		15
		19	29	I		20
	5	20	15	29	I	17
		20	15	I		13
L_3	9	19	36			21
		19	37			22
D_3	9	48				29
		48				29
L_5	9	33	23	I		22
			27	I		23
	9	20	19	29	I	19
			19	I		16
Shortest time	5	12	11	13		
Average time	14	24	22	23		

Table 2.8: Failed Cultures for First Round of Isolation (65°C)

Cultures	Discarded due to
	I - incomplete filter paper degradation
L_1, L_2, L_4, D_4	No filter paper degradation
D_5	>35 days for full degradation
	Discontinued culture

2.3.4 Selection of Mesophilic Consolidated Consortium

(a) Selection of Cellulose Degrading Bacterial Consortia from Second Round of Winogradsky Leachate Inoculations

At least one of each leachate inoculated culture performed in triplicate started degrading the filter paper after seven days, shown in table 2.9. The other cultures took longer than seven days and were discontinued. After sub-culture D_2, D_3 and L_5 were the fastest degraders. $D_{2i\ \alpha iii}$, $D3_{ii\ \alpha iii}$, and $L_{5i\ \alpha ii}$ were selected to be incorporated into the final mixed consortium.

(b) Selection of Cellulose Degrading Bacterial Consortium from First Round of Winogradsky Leachate Inoculations

CD_{2i}, Table 2.9 (b), from the first round of Winogradsky inoculations started degrading filter paper after 22 days, which was comparable to the sub-cultures from round two and was thus selected for consolidation into the final mixed consortium.

(c) Selection of Cellulose Degrading Bacterial Consortia from Termites

A termite culture T1αv, Table 2.9 (c), was selected to be part of the final mixed consolidated consortium.

d) Consolidation of Cultures

Fastest filter paper degrading cultures from the second round of isolation experiments were consolidated for the inoculation of the mesophilic bioreactor. 5ml of each culture were consolidated into a single serum vial:

Final Mesophilic Mixed Consortium = $D2_{i\alpha iii} + D3_{ii\alpha iii} + L5_{i\alpha ii} + CD_{2i} + T_{1\alpha v}$.

2.3.5 Selection of Thermophilic Consolidated Culture

(a) Selection of Cellulose Degrading Bacterial Consortia from Second Round of Winogradsky Leachate Inoculations

After inoculation, a D_2, L_3, L_5 and both D_3 cultures degraded the filter paper in 9 days. After the first sub-culture, all the mixed cultures took longer than nine days to degrade the filter paper. The fastest filter paper degradation times were 5, 12, 15 and 13 days. These were not, however, found to occur in the same lines of subculture. One reason for this may be that some critical bacterial species were lost during sub-culture.

Table 2.9: Second Round of Isolation (37°C)

	Cultures	Start of Degradation (Days)	Full Degradation (Days)	Selected for Sub-culture	Selected for Consolidation
Winogradsky Inoculation	D_{1i}	7		→	
	D_{2i}	7		→	
	D_{2ii}	7			
	D_{3ii}	7		→	
	L_{3ii}	7		→	
	L_{5i}	7		→	
Winogradsky Subculture	$D_{1ii\ \alpha ii}$	25	> 25		
	$D_{2i\ \alpha iii}$	19	> 25		$D_{2i\ \alpha iii}$
	$D_{3ii\ \alpha iii}$	22	> 25		$D_{3ii\ \alpha iii}$
	$L_{5i\ \alpha i}$	6	25		
	$L_{5i\ \alpha ii}$	4	22		$L_{5i\ \alpha ii}$
	$L_{5i\ \alpha iii}$	6	25		
Best Winogradsky culture from round 1	CD_{2i}	22			CD_{2i}
Termite Inoculation	Termite $_1$		43	→	
	Termite $_2$		> 43		
Termite Subculture	$T_{1\alpha i}$		42		
	$T_{1\alpha ii}$		42		
	$T_{1\alpha iii}$		42		
	$T_{1\alpha v}$		42		$T_{1\alpha v}$

(b) Selection of Best Cellulose Degraders from the First Round of Winogradsky Leachate Inoculations

The cultures from the first round of Winogradsky inoculations that remained viable all started degrading filter paper after 13 days. Two of the cultures, CL_{3i} and CL_{5iii}, were selected for consolidation.

(c) Selection of Cellulose Degrading Bacterial Consortia from Termites

The Termite $_1$ inoculum, Table 2.10, started degrading (10 days) faster than the Termite $_2$ culture (more than ten days) and was selected for subculture. $T1_{\alpha i}$ was the only subculture that started degrading in 25 days and was thus chosen to be part of the consolidated consortium.

(d) Consolidation of Cultures

Fastest filter paper degrading cultures from the second round of isolation experiments were consolidated to obtain a consolidated thermophilic consortium. 5ml of each culture were consolidated into a single serum vial:

Final Thermophilic Consolidated Consortium = $D_{1ii\ \alpha i} + D_{2i\ \alpha i} + {}_{D2i\ \alpha iii}$, $L_{5iii\ \alpha ii} + CL_{3i} + CL_{5iii} + T_{1\alpha i}$

2.3.6 Ethanol Production

It is essential to select a consortium that degrades cellulose fast and produces high levels of ethanol. Therefore, ethanol concentration was measured from the Winogradsky and termite cultures that degraded filter paper wholly and efficiently. In the previous section, it was shown that thermophilic cultures degraded faster than mesophilic cultures. The ethanol concentrations of the thermophilic cultures selected for good degrading ability were found to be higher than mesophilic cultures. The ethanol concentrations of all the cultures measured are shown in figure 2.6. The mesophilic ethanol concentrations were mostly below 20 mg/dL,

whereas all thermophilic ethanol concentrations were all above 20 mg/dL except for one that was 18.19 mg/dL. It is also important to note that many more thermophilic cultures than mesophilic cultures degraded filter paper effectively, indicating that thermophilic cellulose-degrading consortia are hardier than mesophilic consortia.

Table 2.10: Second Round of Isolation (65°C)

	Cultures	Start of Degradation (Days)	Complete Degradation	Selected for Sub-culture	Selected for Consolidation
Winogradsky Inoculation	D_{1ii}	7		→	
	D_{2i}	3		→	
	D_{2iii}	3		→	
	$D3_{ii}$	5		→	
	L_{3i}	10		→	
	L_{5iii}	5		→	
Winogradsky Subculture	$D_{1ii\ \alpha i}$	6			$D_{1ii\ \alpha i}$
	$D_{2i\ \alpha i}$	3			$D_{2i\ \alpha i}$
	$D_{2i\ \alpha iii}$	3			$D_{2i\ \alpha iii}$
	$L_{5iii\ \alpha iii}$	6			$L_{5iii\ \alpha iii}$
Best Winogradsky cultures from round 1	CL_{3i}	13			CL_{3i}
	CL_{3ii}	13			
	CL_{3iii}	13			
	$CL5_{iii}$	13			CL_{5iii}
Termite Inoculation	$Termite_1$		10	→	
	$Termite_2$		> 10		
Termite Subculture	$T_{1\alpha i}$		25		$T_{1\alpha i}$
	$T_{1\alpha ii}$		> 25		
	$T_{1\alpha iii}$		> 25		
	$T_{1\alpha v}$		> 25		

Thermophilic ethanol concentrations were consistently higher than mesophilic, except for the lowest thermophilic ethanol concentration of 18.19, which was lower than the two highest mesophilic ethanol concentrations. This is clearly illustrated in figure 2.7 (a), which is a bar graph showing the ethanol concentrations in ascending order. The maximum thermophilic ethanol concentration (74.9 mg/dL) was more than three times the amount of the maximum mesophilic (21.4 mg/dL) concentration. The lowest thermophilic ethanol concentration (18.19 mg/dL) was almost double the lowest mesophilic level (9.32 mg/dL). Significantly higher ethanol concentrations are produced under thermophilic culture conditions than mesophilic conditions.

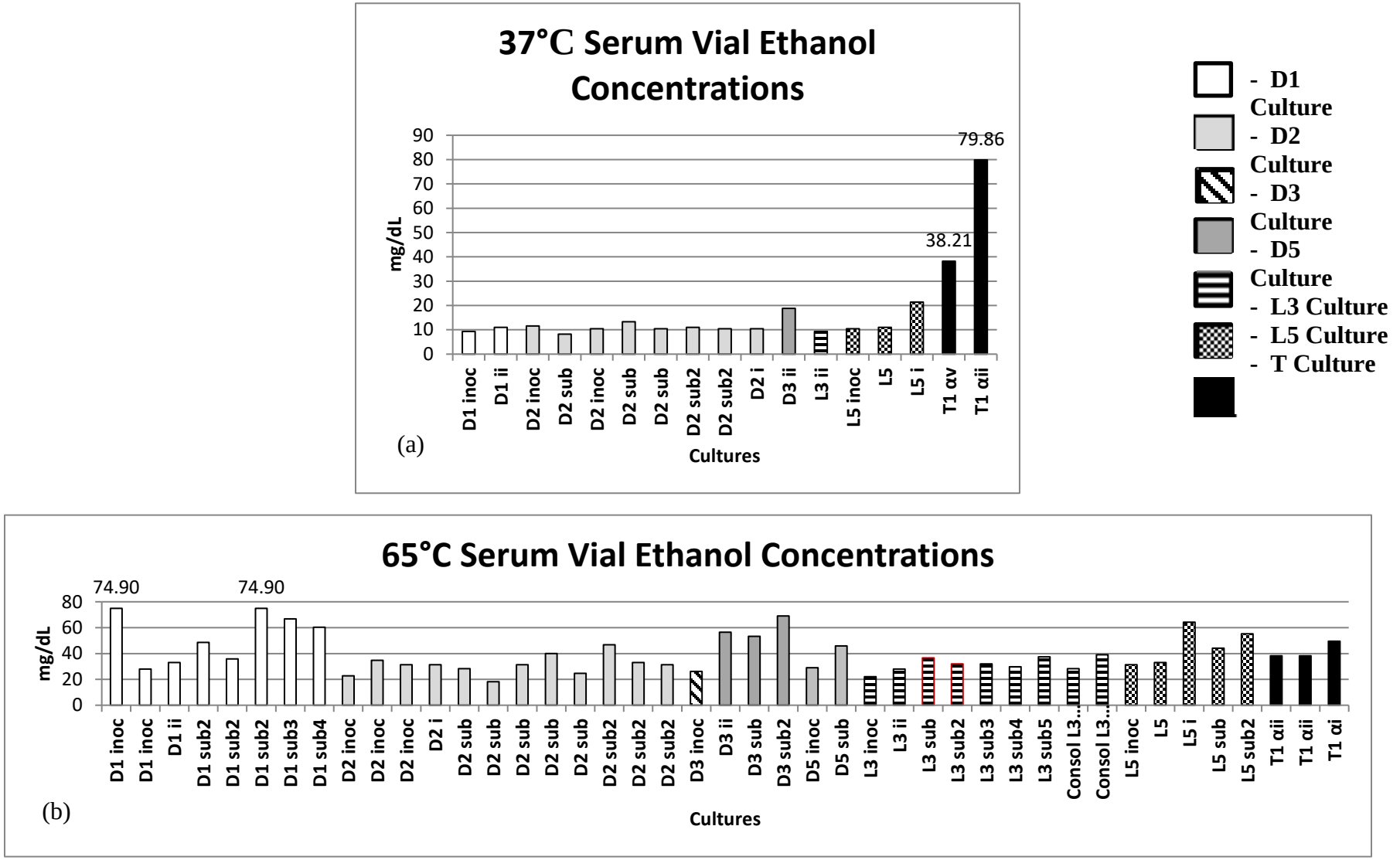
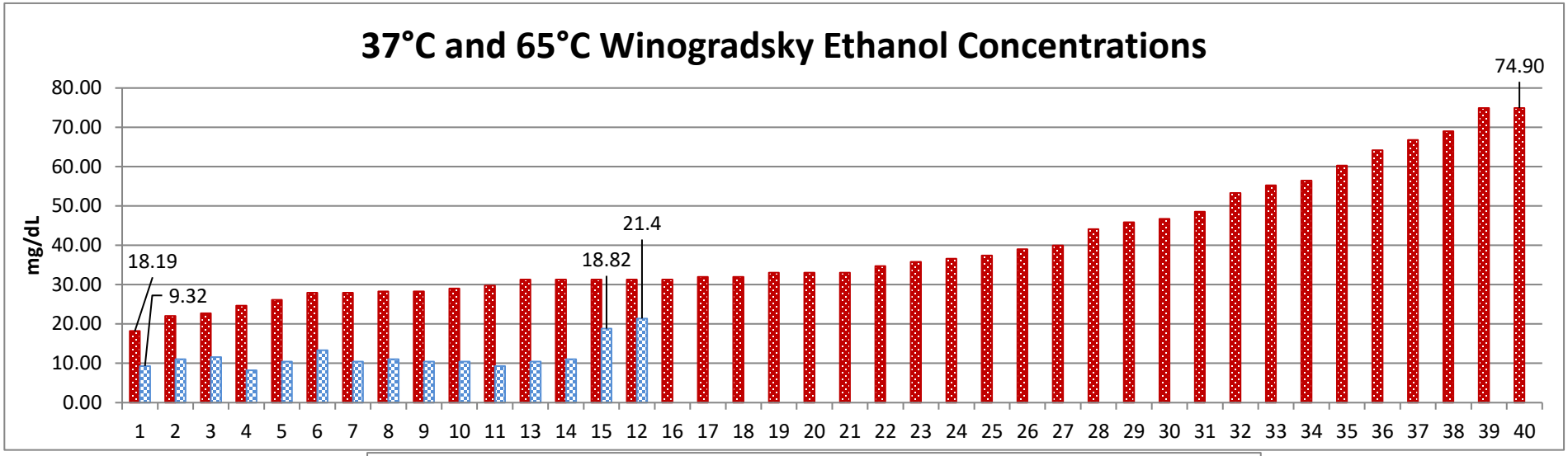
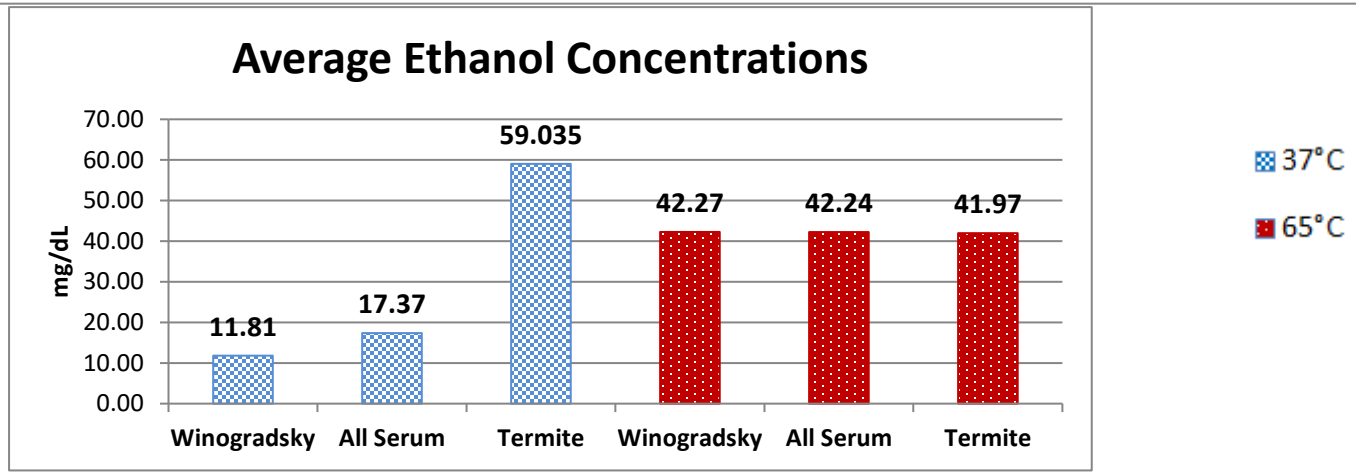


Figure: 2.6: Graphs showing ethanol concentrations produced in serum vials from the first round of Winogradsky and Termite inoculations incubated. (a) Shows the mesophilic cultures incubated at 37°C. (b) Shows the thermophilic cultures incubated at 65°C.



(a)



(b)

Figure 2.7: (a) Ethanol concentrations from 37°C and 65°C cultures arranged in ascending order. (b) A comparison of the average mesophilic and thermophilic ethanol concentrations produced.

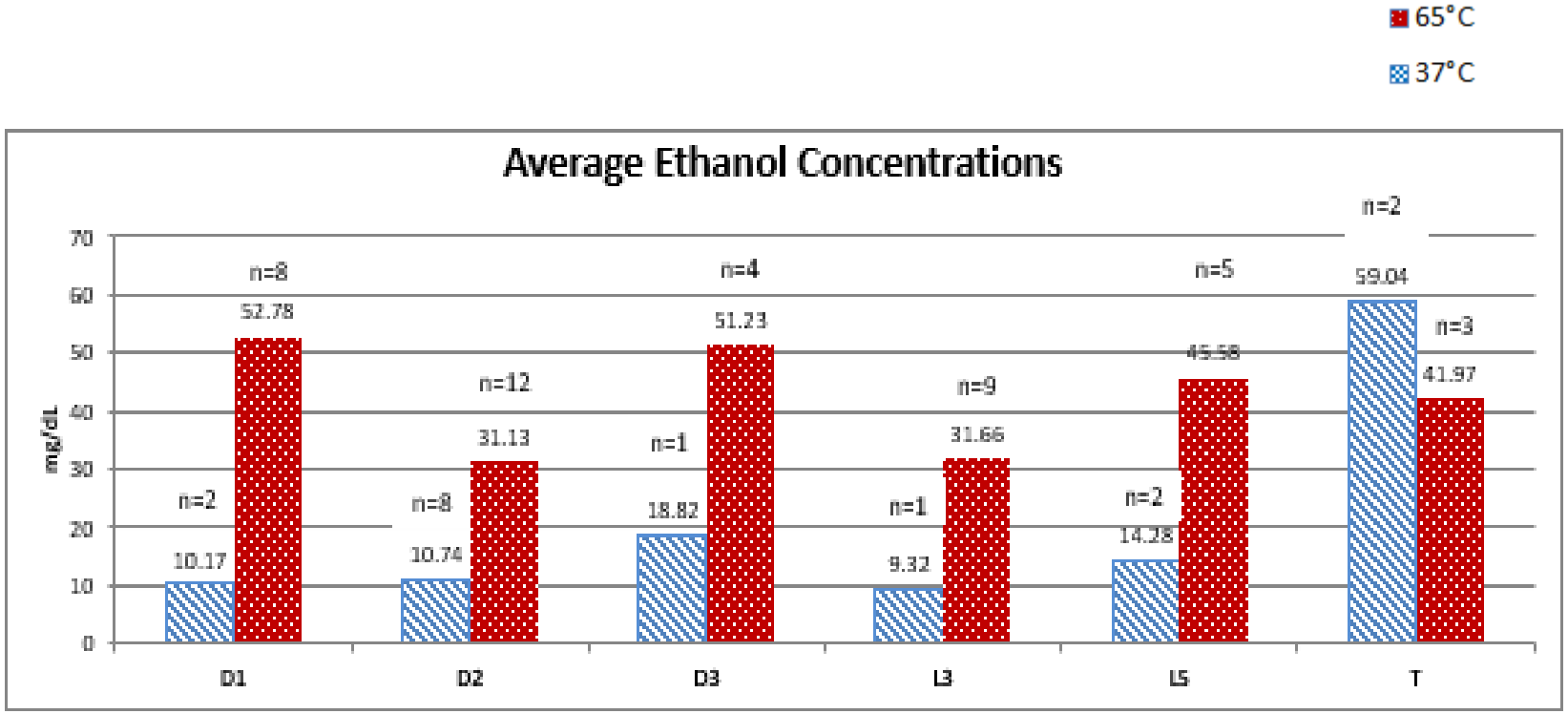


Figure 2.8: Average ethanol concentrations for Termite and Winogradsky round 1. Serum cultures D_1, D_2, D_3, L_3, L_5 and termite cultures that degraded filter paper are entirely compared.

Figure 2.7 (b) compares the average mesophilic and thermophilic ethanol concentrations of the Winogradsky and Termite cultures. The average thermophilic Winogradsky ethanol concentration was significantly higher than the mesophilic. The exception to this was the mesophilic termite concentration (58.035 mg/dL), which was much higher than the average mesophilic ethanol concentration in the Winogradsky cultures (11.81 mg/dL). The high termite values skew the mesophilic results so that it may appear that the mesophilic results were better than the thermophilic results. Because of this, the termite ethanol concentrations were addressed separately to the Winogradsky. The thermophilic termite concentrations, as can be seen in were like the Winogradsky concentrations. The average termite (41.97 mg/dL), Winogradsky (42.27 mg/dL) and all serum (42.24 mg/dL) thermophilic concentrations were all very similar. The high mesophilic termite ethanol concentrations produced is very unusual and unexpected, especially since the peak mesophilic concentration was higher than the thermophilic.

In general, however, the ethanol produced under thermophilic conditions was more than double that produced under mesophilic conditions, even with the same starter inoculum. Figure 2.8 shows the mesophilic and thermophilic lines that were selected for ethanol measurement. The cultures with the starter inoculum: D_1, D_2, D_3, L_3 and L_5 that were incubated under mesophilic as well as thermophilic conditions degraded filter paper most efficiently. The thermophilic cultures produced more than double and sometimes tripled the ethanol levels compared to those incubated under mesophilic conditions, Figure 2.8. Sample D_1 produced the highest average concentration of ethanol (52.78mg/dL) and had the peak ethanol production of 74.9mg/dL in two cultures. Sample D_2 produced the lowest average ethanol concentration but had the highest number of cultures selected (n=12) that remained viable the longest. Sample D_3 produced the second-highest average concentration of ethanol but had the least number of samples, i.e., n=4. D_1 was concluded to be the best starter inoculum for thermophilic cellulose degradation and ethanol production.

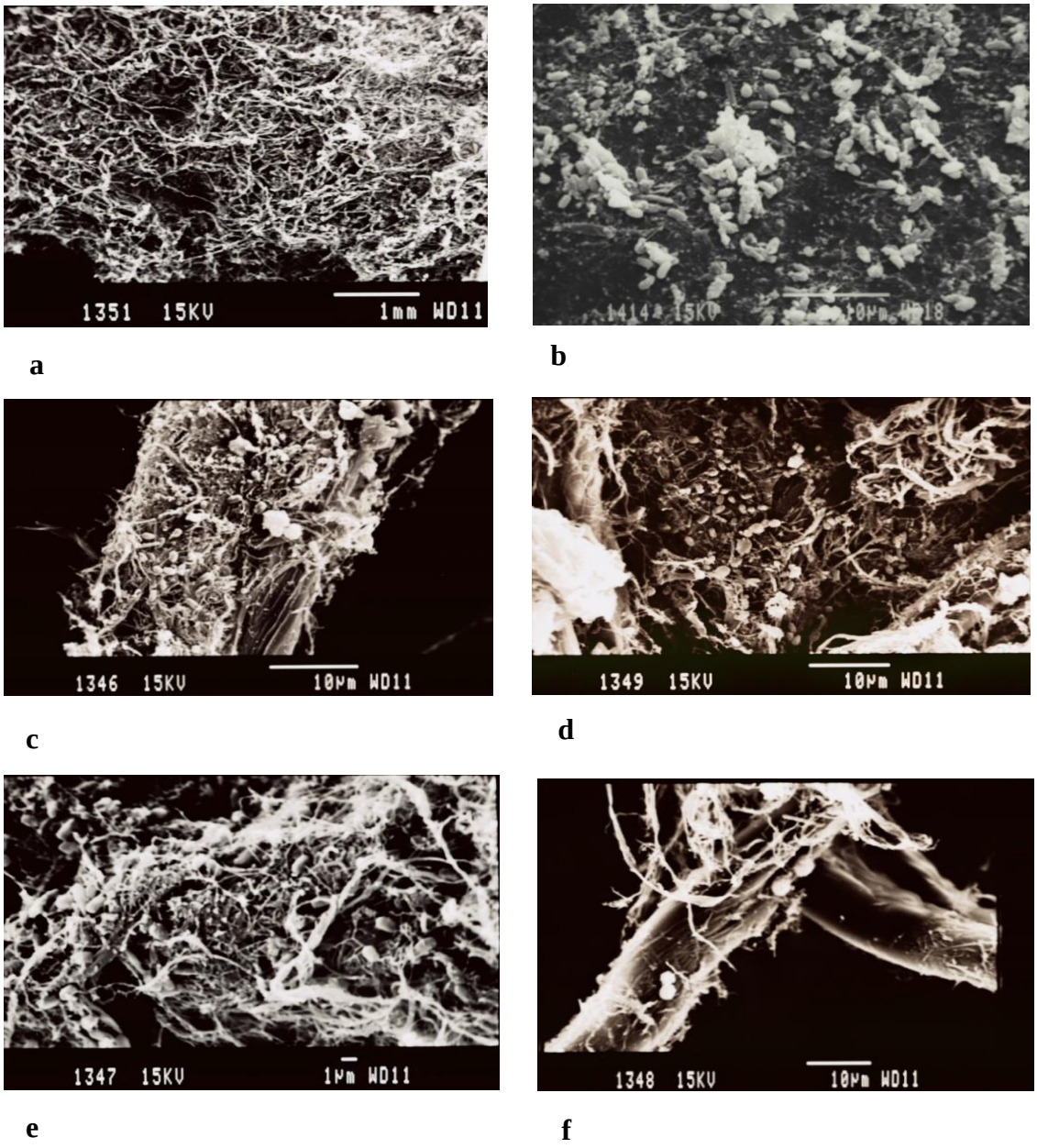


Figure 2.9: Scanning Electron Micrographs of Serum Vial Cultures

(a) Uncultured filter paper. (b) Planktonic bacteria were mainly rod-shaped. (c) Bacteria attached to a strand of cellulose. (d) Bacteria attached by GAC to cellulose fibres. (e) A loose association of cellulose fibres and bacteria. (f) Budding of a coccus shaped bacterial cell on the surface of a strand of cellulose.

There were fewer mesophilic than thermophilic cultures that degraded cellulose well. Sample D_3 produced a unique culture with a peak mesophilic ethanol concentration of 18.82mg/dL. The lowest average ethanol concentration was D_1. D_2 had an average ethanol concentration of 10.74, with a sample size of 8. The other

cultures combined (D_1, D_3, L_3 and L_5) had n= 6, which was less than the number for D_2. For this reason, D_2 was found to be the best mesophilic starter culture.

2.3.7 Scanning Electron Microscopy (SEM)

SEM was performed on the fasted thermophilic inoculated culture, D_{2ii}, that took five days for full degradation. There were different sized rod and cocci shaped bacteria. Bacteria were both planktonic (Figure 2.9 (b) and attached (c) and (d) to cellulose. Planktonic bacteria were mainly rod-shaped. A loose association of bacteria (e) with filter paper was observed, where bacteria were not attached. (f) shows a cocci bacterium budding, while connected to the surface of filter paper.

2.4 CONCLUSIONS

The use of the anaerobic hood and Hungate techniques to maintain strict anaerobic conditions did not yield good results. This experiment showed that the anaerobic could successfully be used to isolate thermophilic sulphate, reducing bacteria.

The second experiment confirmed the success of the Hungate technique and highlighted the problem of using an expired reducing agent. It was thus presumed that the expired reducing agent was responsible for inhibition of anaerobic growth.

A microaerophilic culture technique using Winogradsky column leachate was the method found to be best suited to isolate a microaerophilic bacterial consortium that degrades cellulose and produces ethanol

The heat kill step ensured that all vegetative cells were killed, and only spores remained. Facultative anaerobic spores present would be able to germinate in the presence of oxygen to use the remaining oxygen before switching over to anaerobic metabolism (Haruta, 2002). Only once anaerobic conditions were created by the facultative anaerobes, could the obligate anaerobes sporulate. Both facultatively anaerobic and anaerobic cellulolytic bacteria are required to develop a stable consortium for successful maintenance of cellulose degradation, as confirmed by

Haruta (2002). The loss of facultative anaerobes from the consortium was thought to have prevented anaerobic cellulolytic degradation of cellulose.

Cellulose degradation was almost always the fastest after the initial inoculation. After subculture degrading ability was eventually lost, this may have been due to the microaerophilic subculture techniques employed.

A second Winogradsky leachate inoculation was performed and selection made from the inoculated cultures instead of after subculture, to bypass the loss of degrading ability. A final mixed consortium was selected from a few of the cultures that started degrading fastest to obtain the most diverse spectrum of facultative anaerobes and obligate anaerobes possible. The selected mixed consortium would then stabilise and adapt to conditions in the bioreactor (Chapter 3).

CHAPTER 3
ANAEROBIC FLUIDISED BED REACTOR

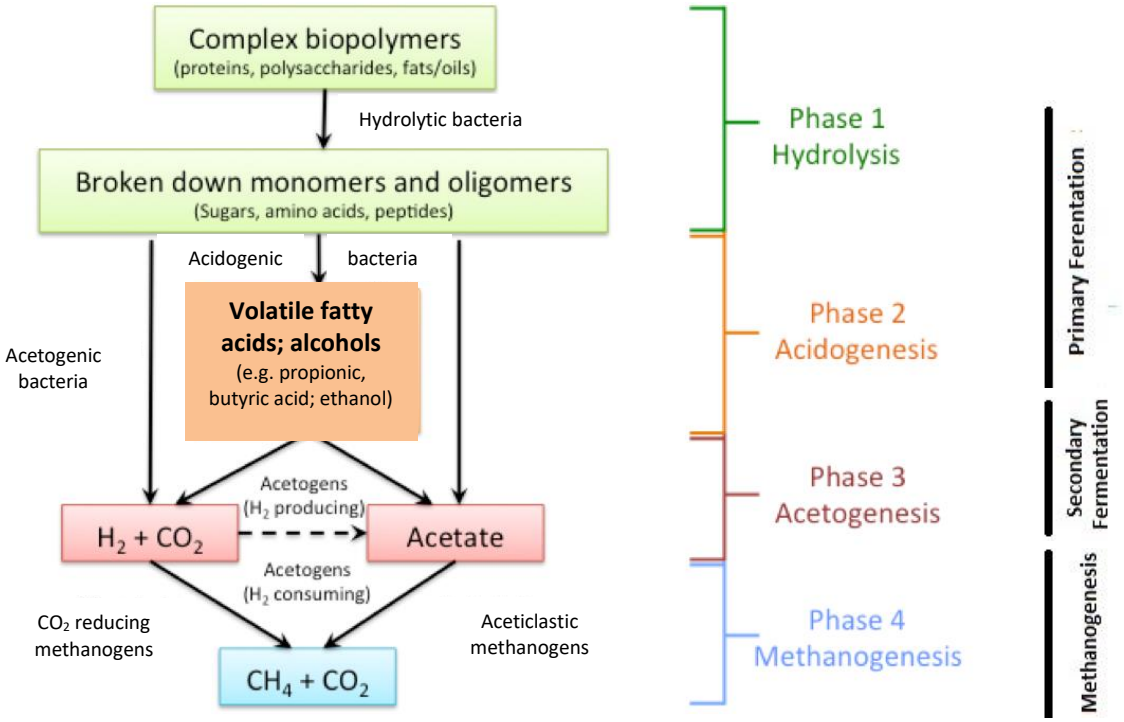
3.1 INTRODUCTION

Dwindling petroleum-based fuels and frightening environmental effects has stimulated the emergence of lignocellulose-based renewable alternatives. Lignocellulose is the most abundant renewable feedstock with enormous potential to produce biofuels, bio-based chemicals, and materials for a sustainable energy future (Mukherjee *et al.*, 2017).

Wheat bran, a by-product of the wheat milling industry, continually generates enormous surpluses. It may present a unique opportunity for the utilisation of surplus Agricultural wastes such as surplus wheat bran as a feedstock for the generation of renewable sources of energy via non-fossil routes (Hawkes *et al.*, 2008). Multiple co-products in a biorefinery make the process attractive and more economically viable (Angelidaki, 2007; Raposo *et al.*, 2016).

Biofuels in the form of ethanol, hydrogen, and methane are products of the dark anaerobic digestion of cellulosic substrates such as wheat bran. From an engineering point of view, it is preferable to produce biofuels from a continuous dark anaerobic process (Solowski, 2018). One possible configuration for a continuous dark anaerobic bioreactor is in the form of a dark anaerobic fluidised bed bioreactor where the wheat bran performs the dual function of being both the supplier of the carbohydrate feedstock as well as a substrate for bacterial biofilm attachment, forming granules like those comprising granular sludge. In nature, a multi-species consortium of mutualistic bacteria is required to break down lignocellulosic substrates such as wheat ban to produce volatile fatty acids, alcohol, hydrogen, carbon dioxide, and methane (Cabrol *et al*., 2017). The consortium of bacteria that generally degrade complex carbohydrates such as proteins and fats, as well as carbohydrates, include the following metabolic functional groups: hydrolysing bacteria, acidogenic bacteria, solventogenic bacteria and methanogenic

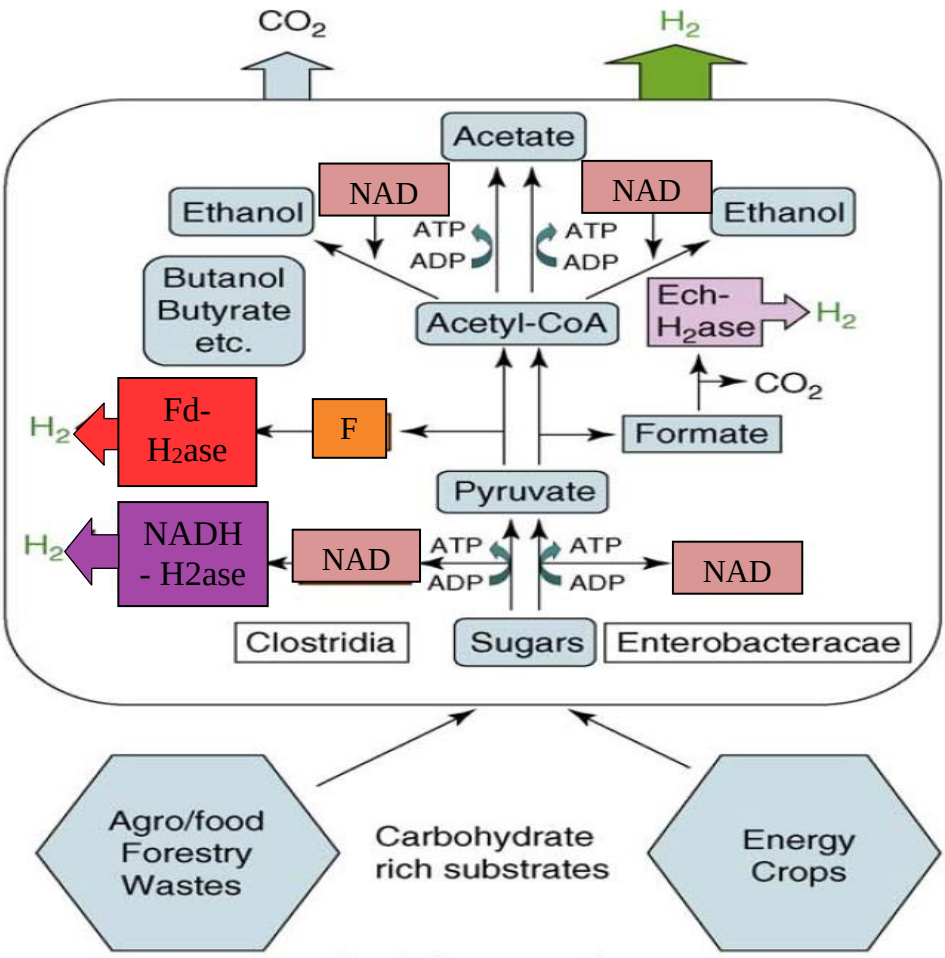
bacteria (Figure 3.1). The hydrolysing bacteria would comprise anaerobic cellulolytic bacterial species (Gaudy, 1981). Such a fluidised bed could be operated as either a mesophilic or thermophilic system.



(Clifford, 2018)

Figure 3.1: Schematic diagram showing four phases of biogas production.

The production of ethanol from a cellulosic substrate such as wheat bran using an immobilised multi-species bacterial consortium appears in principle to be achievable given the nature of dark anaerobic metabolism and the products generated by this process. Figure 3.1 shows that hydrogen (H_2) and is an essential intermediate in the dark anaerobic oxidation of organic compounds to form the end products CO_2 and CH_4 (Clifford, 2018). In summary, from Figure 3.4, a dark anaerobic heterotrophic bioprocess would simultaneously comprise an acidogenic and solventogenic stage. In the acidogenic step in acetic, propionic and butyric acids plus hydrogen would be produced. In the solventogenic stage, ethanol and possibly butanol could be generated.



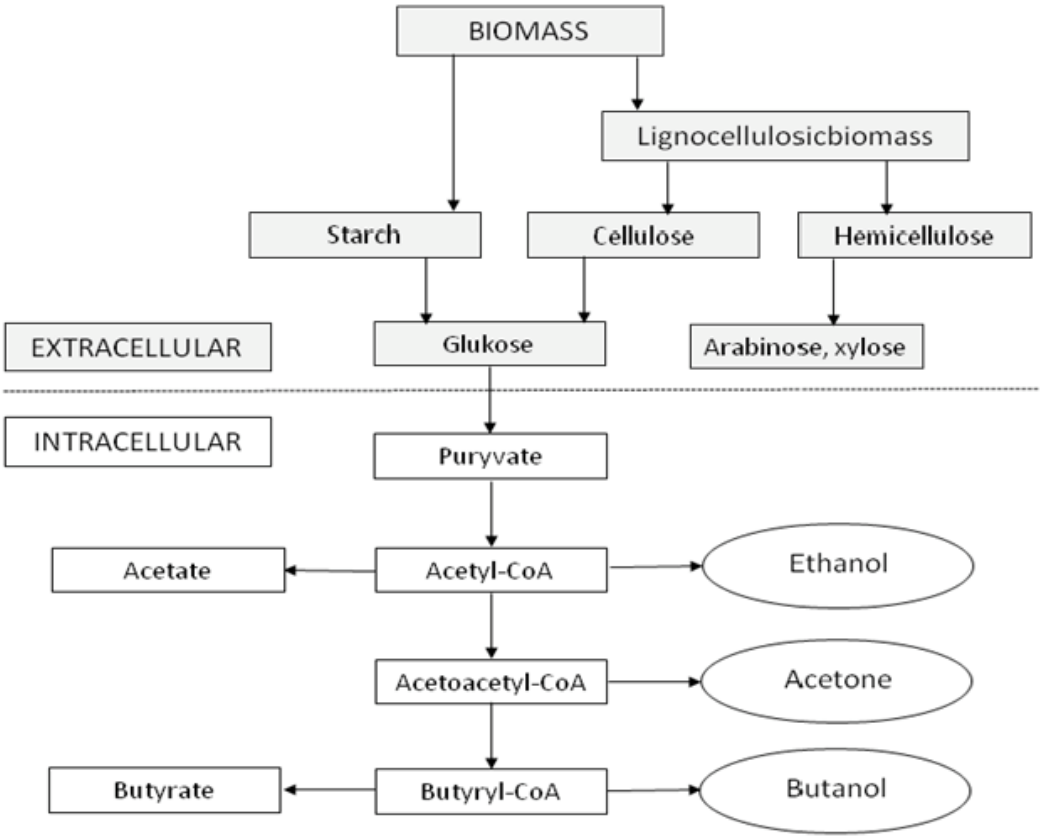
(Hallenbeck and Gosh, 2009)

Figure 3.2: Dark heterotrophic solvent and hydrogen production

It should be noted from Figure 3.1 that dark anaerobic oxidation of complex organic compounds such as cellulosic substrates (wheat bran) depends on the cooperative metabolic interactions between the following guild of bacteria:

1. The primary fermentation bacteria include hydrolytic bacteria that break down complex molecules like lignocellulose to monomeric glucose, xylose, and arabinose. Acidogenic bacteria utilise these simple sugars to produce volatile fatty acids and alcohols.
2. The secondary proton reducing syntrophic or acetogenic bacteria produce acetic acid from acetate. They can oxidise short-chain fatty acids such as butyrate and propionate to acetate and can also oxidise acetate to CO_2 and H_2.

3. There are also acetogenic bacteria that produce acetate from CO_2 and H_2 or the oxidation of butyrate and propionate.
4. Two types of methanogenic archaea bacteria (Gaudy and Gaudy, 1981).



(Matuszewska, 2016)

Figure 3.3: A global overview of a biofuel generation in the dark anaerobic heterotrophic fermentation process.

Dark heterotrophic H_2 and solvent production (Figure 3.2) is carried out by a wide variety of facultative and obligate anaerobic bacteria involving two alternative bacterial hydrogen-producing enzyme systems. Ferredoxin Fe-Fe dependent hydrogenase pathway – Clostridial type fermentation system, which includes the ethanol and hydrogen-generating obligate anaerobes *C. butericum, C. pasteurianum, C. cellulolyticum, C. thermocellum*. The other pathway uses Ech-H_2ase (NiFe) hydrogenase – Enterobacterial type fermentation system: H_2 generating facultative anaerobes: *Escherichia coli, Enterobacter aerogenes, E. cloacae, Citrobacter freundii, C. intermedius, Klebsiella pneumoniae* (Hallenbeck and Gosh, 2009; Ghimire *et al.*, 2015).

Furthermore, the primary fermentation bacteria include hydrolytic bacteria that degrade the various biopolymers such as proteins, polysaccharides (starch and cellulose), nucleic acids, and lipids to monomers such as amino acids, sugars, purines, pyrimidines, long-chain fatty acids. Acidogenic bacteria: This group of primary fermentation bacteria ferments the monomers to alcohols, short-chain fatty acids (acetate, butyrate, and propionate), organic acids, CO_2, and H_2 (Gaudy, 1981).

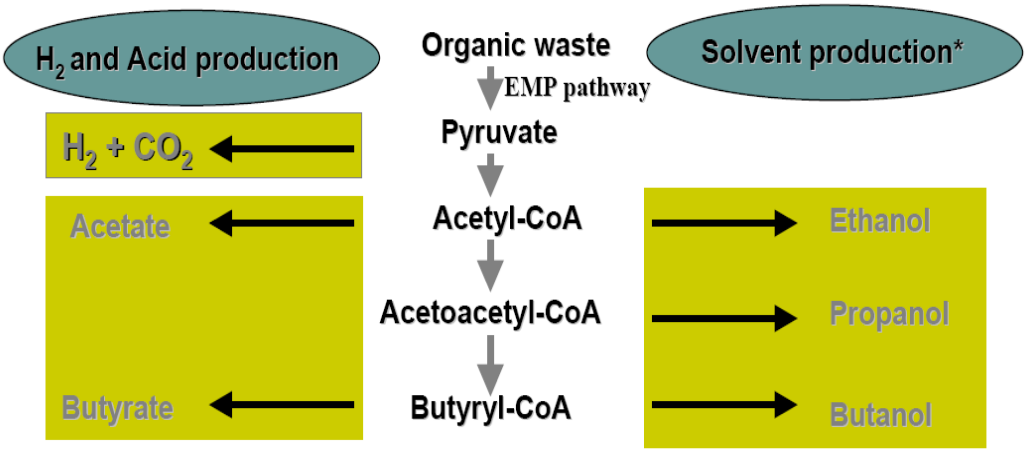


Figure 3.4: Schematic diagram showing the two opposing pathways in the dark fermentation of organic waste: the solventogenic and hydrogen/acid-producing pathways.

A dark fermentation process will encounter acidity problems: For example, acidogenic bacteria can produce fatty acids faster than their consumption rate by the syntrophic bacteria, acetogenic bacteria, and methanogens. Acid inhibition of methanogenesis results when the pH falls to below 7.0. Therefore, operating the anaerobic bioreactor below pH 7.0 may also shift electron flow from methanogenesis to solventogenesis and acidogenesis (Cabrol *et al*., 2017). The main pathway would also need to be oriented toward solventogenesis (acetone, butanol end ethanol production) and away from acidogenesis (hydrogen-producing acetobutyric pathway), shown in figure 3.4, using the same thermoanaerobic bacteria.

3.1.1 Anaerobic Fluidized Bed Bioreactor

The global biochemistry illustrated in Figure 3.4, that is embodied in the metabolic cooperation of a multi-species dark anaerobic bacterial consortium degrading lignocellulosic biomass within the fluidized bed of a bioreactor strongly suggests that the co-production of ethanol and hydrogen are possible, and the bioreactor can generate both a gas stream containing hydrogen and an effluent stream containing ethanol. The multispecies consortium of bacteria would either exclude or include methanogenic bacteria but would necessarily have to include hydrolytic, acidogenic, syntrophic volatile fatty acid oxidising bacteria and solventogenic bacteria. In this study, the exclusion of methanogenic bacteria was required to restrict the pathway to ethanol and hydrogen production. Methanogens were eliminated from the starter consortia by the heat treatment of environmental samples to kill all vegetative growth, excluding spores.

Several different bioreactor configurations have been used for ethanol and hydrogen production using a wide range of substrates, including lignocellulosic biomass. Most studies have been carried out in batch continuous stirred tank reactor (CSTR), fixed or packed bed reactors, up-flow anaerobic sludge bed (UASB) reactors, plug flow reactors or membrane bioreactors (MBR). Recent research has focused on semi-dry (10-20% total solids) or dry (>20% TS) (Ghimire *et al*., 2015). The focus of this research was to conduct a preliminary study testing the possibility of using AFBR was for the mesophilic and especially thermophilic dark fermentation of a solid lignocellulosic, i.e., bran as a feedstock for ethanol production. Optimisation of parameters was not a part of this study, only a proof of concept, i.e., the production of ethanol/hydrogen and the degradation of the lignocellulosic substrate, i.e., bran.

In the development, operation, and optimisation of a continuous dark anaerobic fluidised bed bioreactor for the co-production of hydrogen and ethanol, there are many considerations. As has already been stated, the insoluble cellulosic substrate in the form of wheat bran would serve the following two bioprocess functions: 1) provision of the primary nutritional source of carbon, and 2) provision of a solid

substrate for bacterial attachment and biofilm formation. In the design, development, and operation of the bioreactor process, the bed of bran must be maintained in a suspended state within the bioreactor by the recycling part of the effluent, which passes through the gas disengager back into the bioreactor (see Figure 3.6). A constant volume of the liquid phase must be maintained in the bioreactor to allow the excess amount due to influent flow to be decanted from the bioreactor system via the gas disengager. This process would facilitate the continuous removal of hydrogen from the effluent recycle stream. Also, at thermophilic temperatures, ethanol would be removed from the bioreactor by evaporation within the gas disengager primarily if the gas disengager were operated at high temperatures. In this study, we measured ethanol in the effluent stream and hydrogen from the gas disengager.

A critical technical problem associated with the operation of the bioreactor as a continuous process involved the supply of wheat bran to the bioreactor. Feeding rates which would maintain the density or concentration of wheat bran within the bioreactor at a constant state so that the process would operate as a chemostat was challenging.

The technical feasibility regarding an anaerobic fluidised bioreactor process for generating ethanol from a solid substrate such as wheat bran a set of criteria was defined. The accomplishment of these objectives established the proof of concept regarding the bioprocess. Proof of concept would involve the following: 1) a steady reduction in the settled bed volume during the batch feeding intervals, indicating cellulolytic degradation of the fluidized bran bed, 2) presence of glucose in the effluent stream will confirm the formation cellulose hydrolysis, 3) generation of biohydrogen will confirm that some of the electrons derived from glucose catabolism had gone into proton reduction, 4) the presence of ethanol in the decanting effluent stream will confirm that electrons derived from glucose would have gone into solventogenesis. The theoretical possibility also exists for syntrophic oxidation of volatile fatty acids to hydrogen, and consequently, ethanol could be driven by efficient liquid-gas phase partitioning of hydrogen in the absence of methanogens. The conditions for this possibility may arise because of active

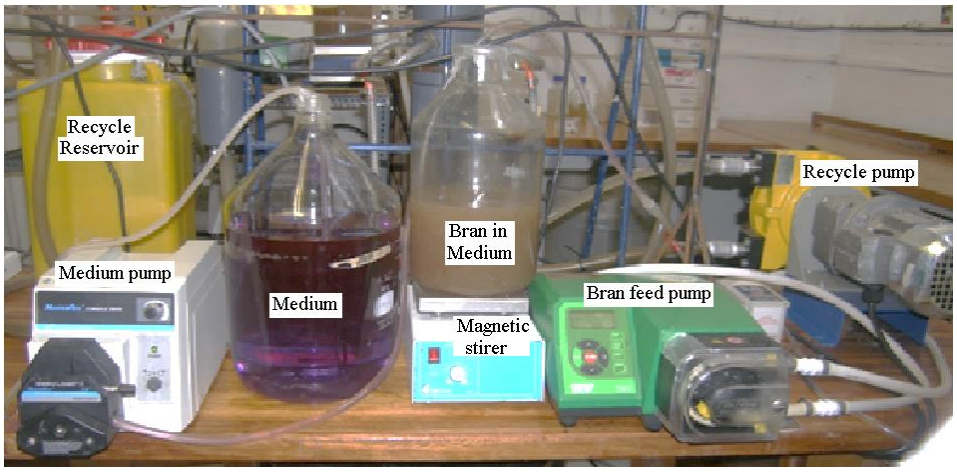
liquid-gas phase partitioning in the fluidised bran due to degassed effluent recycling.

3.1.2 Biofilm and Granule Formation

In nature, microbial consortia are very often found in a surface-attached conformation known as a biofilm the close interactions among bacterial species comprising a biofilm play an essential role for consortia produce biofuel. Biofilms act as a form of protection as well as to decrease diffusion requirements (Wang and Chen 2009). The close association of substrate proximity in biofilms allows for effective cellulose hydrolysis and provides a competitive advantage over non-attached microorganisms. It has also been shown that biofilms enhance the inherent health and robustness against adverse ecological niches, which is an important consideration when producing potentially toxic fuel molecules (Davey and O'Toole, 200). Cell attachment to the substrate in the form of a biofilm can also have the favourable capacity of maintaining elevated microbial biomass levels in bioreactors, which translates to higher ethanol production (Rosche *et al*., 2009). The spatial arrangement generally regulates cooperation within mixed-species biofilms, and the creation of gradients of chemical metabolite could be manipulated to generate zones appropriate for the degradation of lignocellulosic biomass and biofuel production.

Ideally, the self-generating spatial organisation of the biofilm would relieve ethanol inhibition. The development of biofuel producing consortia depends on the metabolic capacity of cooperative partners. Also, advances in techniques for establishing stable and scalable symbiosis as well as a comprehension of scale-up principles for bioreactor systems. The design, management, and conversion of a bioreactor system from laboratory to commercial scale especially impactful since bioprocessing dependant on consortia will also introduce the challenge of limitations to mass transfer (Wang and Chen 2009). (Rosche *et al*., 2009).

(a)



(b)

Figure 3.5: Photograph of mesophilic bioreactor setup (water baths not shown). (a) 1-Anaerobic Fluidised bed reactor, 2-Gas Disengager, 3-Recycle Reservoir, 4-Nutrient Medium, 5-Media Pump, 6-Bran in medium, 7-Magnetic Stirrer, 8-Bran Feed Pump, 9-Recycle Pump. (b) Shows the setup of the peristaltic pumps used for the bran, the nutrient medium feed, and recycle feed.

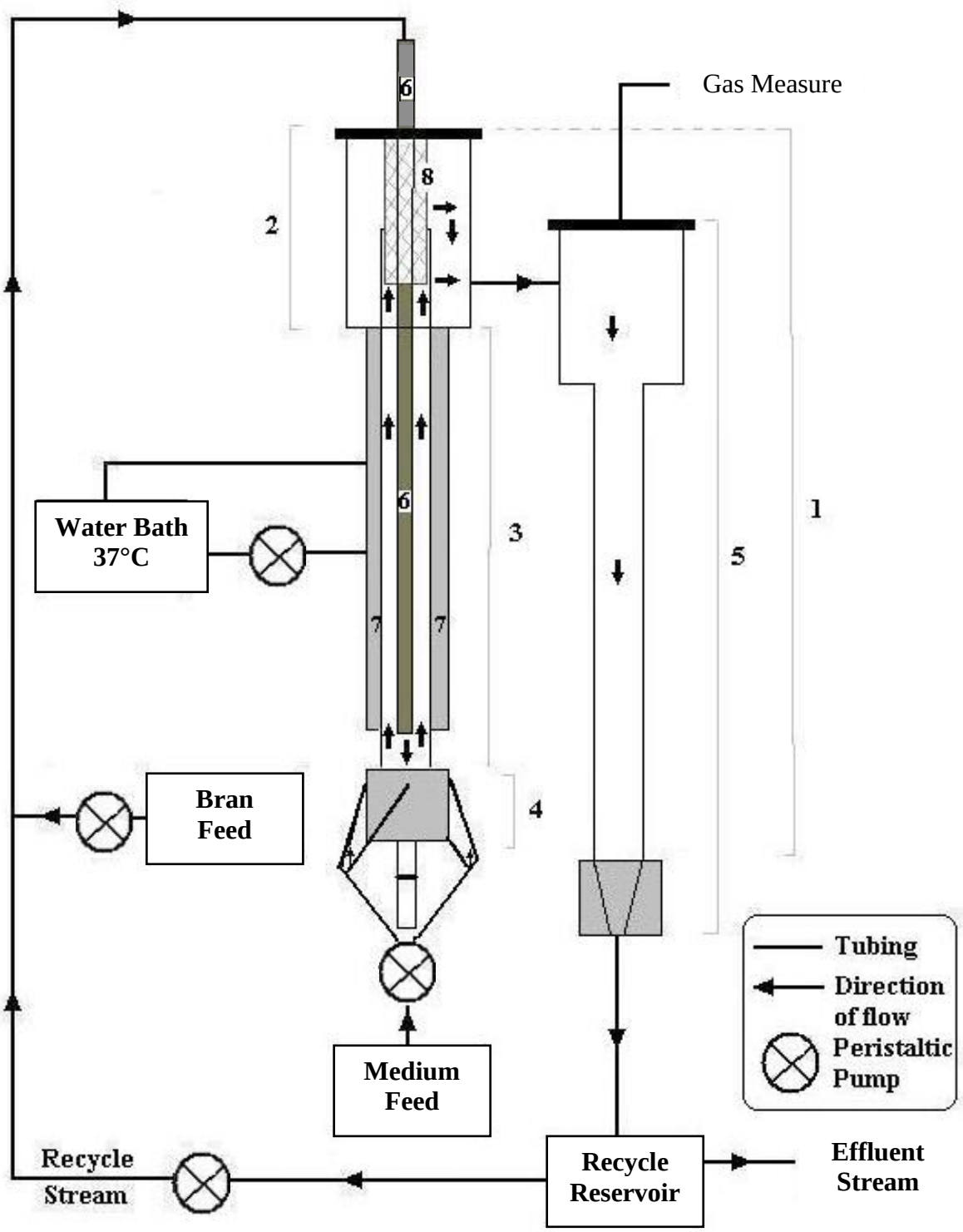


Figure 3.6: Schematic diagram showing the setup of the Mesophilic Bioreactor. [1] Anaerobic AFBR, [2] Solids-liquids separator, [3] Fluidised bed, [4] Base of the reactor, [5] Gas exchanger, [6] Bran Feed and effluent recycle tube, [7] Water jacket, [8] Wire net cylinder.

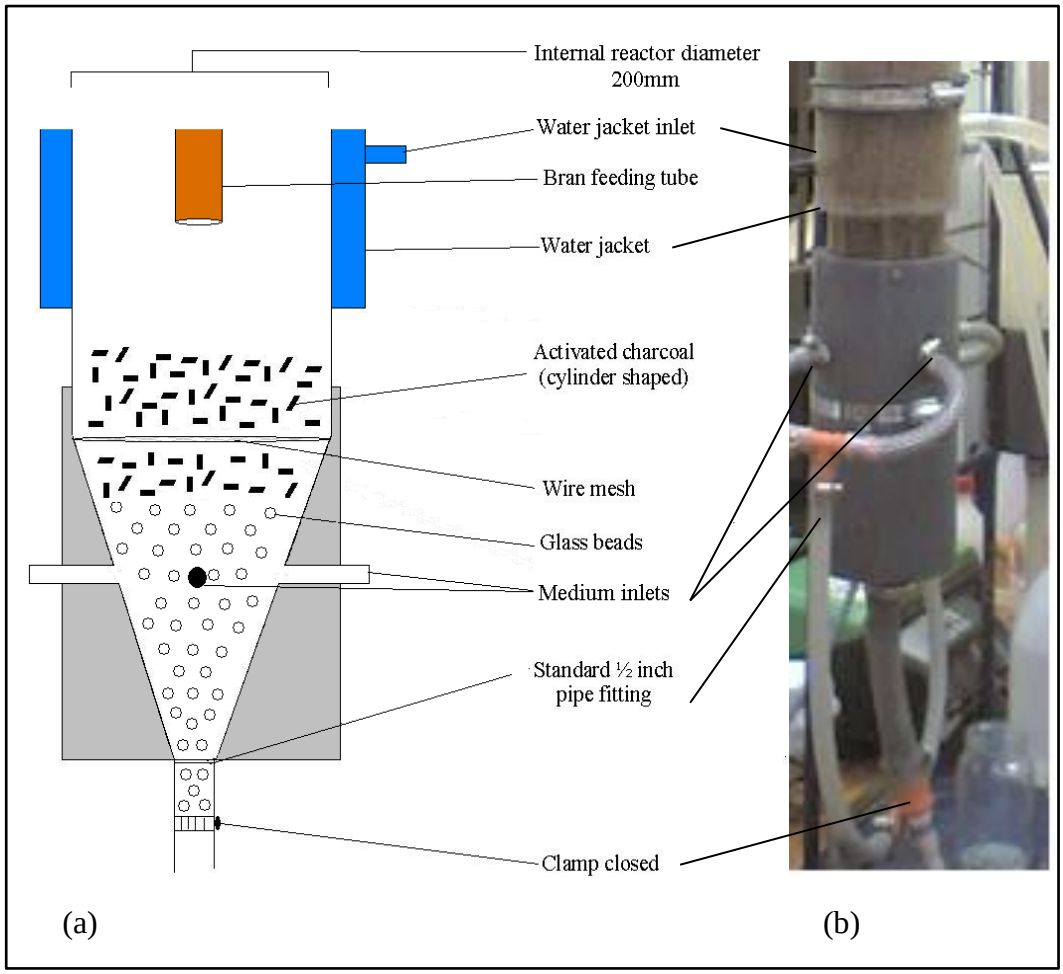


Figure 3.7: **Illustrated diagram of the reactor base, showing the internal diffuser.** The inner components of the reactor base; (a) was fitted with an internal conical-shaped diffuser. The bottom of the diffuser was filled with a layer of glass beads followed by a layer of cylinder-shaped activated charcoal (CAC), which cannot be seen through the grey reactor base. The external view of the reactor base is shown in (b).

Studies have correlated bioethanol production with the concentration of the microbial population and, therefore, alternative cell retention strategies. Immobilisation of ethanol-producing inoculum offers process advantages such as easier handling, high metabolic activity, increases cell density, reuse of cells, better solid/liquid separation efficiency, and better operational stability. There are several immobilisation methods available, which include gel entrapment, ceramics or glass beads, polyacrylamide gels, granulation, and biofilm (Sekoai and Daramola, 2017). The type immobilisation method used was a combination of granulation and biofilm. The biofilm attached directly to the bran substrate forming granules that were kept fluidised.

3.1.3 AIMS

Confirm the cellulose-degrading efficiency of mesophilic and thermophilic consortia.

3.1.4 OBJECTIVES

To operate a mesophilic and thermophilic AFBR system: 1) at a steady reduction in the settled bed volume during the batch feeding intervals, indicating cellulolytic degradation of the fluidised bran bed, 2) confirming the presence of glucose in the effluent stream will confirm the formation cellulose hydrolysis, 3) the presence of ethanol in the decanting effluent stream will confirm that electrons derived from glucose would have gone into solventogenesis. 4) generating of biohydrogen to verify that some of the electrons derived from glucose catabolism had gone into proton reduction,

3.2 MATERIALS AND METHODS

3.2.1 Mesophilic Bioreactor

(a) Substrate Pre-treatment

Wheat bran, used as the sole carbon source, was pre-treated by liquid hot water (Mosier *et al.*, 2005; Lynd *et al.*, 2002; Laser *et al*., 2002). Wheat bran was autoclaved in 1 ½ L of medium for the standard 20 minutes at 121 psi before being diluted to 5g/L with the medium.

(b) Nutrient Medium

Modified ATCC 1190 medium (for the culture of *Clostridium thermocellum)* (Appendix B), which was used in the serum vial isolation of the consortium (chapter

2), was used to run the bioreactor. The only modification was that the vitamin solution was excluded from the bioreactor medium. Resazurin was used to indicate when the bioreactor was aerobic and anaerobic, but no reducing agent was added. The medium was made up using $_{dd}H_20$ and autoclaved for 20 minutes.

(c) Bioreactor Inoculum

Mesophilic fluidised bed bioreactor was inoculated with the cellulose-degrading consortium isolated in Chapter 2 from Winogradsky columns and termites.

(d) Bioreactor Setup

The bioreactor designed and developed for the co-production of ethanol and hydrogen from wheat bran shares similar features with the bioreactor developed by Kleijnjens *et al.* (1986). The mesophilic bioreactor was set up, as shown in Figure 3.5, and is further illustrated in the schematic diagram, Figure 3.6. Following Figure 3.6, the bioreactor vessel [1] for the anaerobic digestion of wheat bran was constructed from perspex tubes with a wall thickness of 5mm. The internal diameter was 80mm and the height 600mm. The working volume for the bioreactor's fluidised bacterial granular bed was 3L, and the volumetric productivity of hydrogen was indicated relative to this volume rather than the total working volume of the entire bioreactor system.

The entire bioreactor vessel was inserted into a second Perspex tube with an internal diameter of 110mm, which functioned as a water jacket [7] for maintaining the temperature of the bioreactor vessel. The bioreactor temperature was maintained at 37°C by re-circulating water from a heated water bath through the water jacket.

The base of the bioreactor tube [4] was fitted with a diffuser (ID: 80mm and H: 150mm) composed of solid PVC, which had been machined on a lath. The diffuser consisted of a hollow cone section and a hollow cylindrical section shown in Figure 3.7 (a).

Figure 3.8: Loading of bran to bioreactor and formation of granules

(a) Mesophilic reactor just after it had filled with new bran. (b) and (c) Show the granules that formed after a few days of running the reactor continuously.

The nutrient medium inlet opened into the cone section of the diffuser. Positioned in the middle of the diffuser were four inlet ports (ID 5mm) with each inlet arranged at 90^{o} to the two other inlets on each side. Four openings in the bottom of the Perspex bioreactor tube were aligned with the above inlet ports. A circular stainless sieve (32 mesh) was positioned on the bottom ledge (which formed the rim of the cone) of the cylinder portion of the diffuser, and the bottom

A solid/liquid separator Figure 3.6 [2] was fitted to the top of the bioreactor, which prevented the loss of bran and granules into the gas disengager [5]. The separator had a height of 200mm and an internal diameter of 140mm. A stainless-steel filter cylinder [8] was fixed into position within the solid/liquid separator around the bioreactor's upper outlet opening to prevent solids from escaping from the system

The edge of the Perspex bioreactor tube rested on the sieve covered ledge of the cylindrical portion of the diffuser. A rubber ring seal between the inner diffuser cylinder walls and the outer bioreactor tube wall made the connection between the diffuser and the bioreactor tube watertight. The diffuser contained a 100mm bed of 5mm glass beads overlaid by a 50mm bed of activated carbon cylinders (CAC), supported by the stainless-steel sieve. Nutrient medium (influent stream) was supplied straight into the upper glass bead layer through four inlet ports.

The nutrient medium was pumped into the bioreactor at the selected flow rate using a Masterflex peristaltic pump (easyload model 77200-50). The nutrient medium was fed from the bottom of the reactor, illustrated in Figure 3.7, through medium inlets through the grey covering and flowed up through the glass beads and the activated charcoal. The medium also played a crucial role in buffering the bioreactor pH by reducing the acidity of the liquid phase.

Bran slurry was kept in suspension using a Labcon magnetic stirrer before being added to the reactor, Figure 3.5 (b), before being added to the reactor. Bran slurry was pumped directly into the bioreactor via the Perspex feed tube [6] with an internal diameter of 20mm, which extended through the lid of the solid/liquid separator [2] to the bottom of the bioreactor

Bran was deposited as a slurry down the feed tube [6] down to the conical diffuser at the base of the bioreactor, where it was mixed into the fluidised bran bed which filled the bioreactor by turbulent flow action of the nutrient medium feed from the bottom of the reactor. The fluidised bran was maintained at a constant height of 10 to 15 cm below the bioreactor outlet into the solid/liquid separator.

(a) (b)

Figure 3.9: Gas **Exchanger** (a) and (b) a closer view of the gas bubbles formed at the surface of the effluent.

A Watson-Mallow Bredel (model 520U) peristaltic pump (Falmouth, UK) was used to pump the bran slurry into the bioreactor. Attempts were made to inject the wheat bran as sludge or pulp continuously down the feed tube into the bottom of the bioreactor at a very slow rate so that the fluidised bran bed height in the bioreactor would remain constant. An attempt at doing this was made with a peristaltic pump, but the exercised proved to be inefficient due to pipes becoming clogged up and blocked with wheat bran pulp. Specialised sludge pumps would be necessary for pumping bran pulp into a fluidised bed bioreactor. Because of the bran sludge pumping problems, it was decided to supply wheat bran as a sludge in a batch feed process at set time intervals.

The effluent overflow from the solid-liquid separator was decanted into the liquid-gas separator or gas-disengager [5]. The gas disengager consisted of a gas collection cylinder (H: 200mm and ID: 150mm) connected to a gas-disengager cylinder (H: 600 mm and ID: 60 mm). The gas-disengager had a working volume of 1.54L, and the total fluid occupied a volume of connecting tubing was 0.934L. The entire working volume of the bioreactor system, including bioreactor bed, solid-liquid separator, gas-disengager, diffuser, and tubing, was 5.5L.

Gas was released through a vent in the top of the gas exchanger while the gas-disengager effluent flowed into the recycle reservoir. The lid of the gas disengager contained a gas outlet port from which gas samples were collected. The recycle reservoir had an effluent outlet and a recycle feed that joined the bran feed to recycle through the reactor. Liquid samples were collected from the effluent stream of the recycle reservoir. A variable Boyser® Bonfiglioli AMP-16 peristaltic pump (0.37 kW) was used to recover the de-gassed effluent back into the bioreactor. For effluent recycling, the pump was set at 45 rpm, which gave a volumetric flow rate of 3.2 L/min.

The recycling of effluent from the gas disengager back into the bioreactor via the diffuser facilitated liquid-gas phase partition of hydrogen, seen in Figure 3.9. The gas disengager was responsible for reducing the concentration of hydrogen trapped in the effluent recycle stream to its thermodynamic equilibrium concentration. The gas disengager promoted the maximum release of hydrogen from the liquid within the gas disengager to the gaseous phase. The gas produced was continuously removed through the exhaust port of the gas disengager. Elevated effluent recycling rates between the bioreactor and the gas disengager created a high degree of turbulence and cavitation within the liquid in the gas disengager tube, encouraging the release of hydrogen from the effluent. This dissolved supersaturated hydrogen was thus enabled to be released through bubble production (Figure 3.8 (b). Removal of hydrogen trapped in the effluent by the gas disengager circumvented the building up of pressure in the system, which was responsible for disruptive leaks and burst pipes, thus increasing the stability and efficiency of the bioreactor. The

glass bed within the diffuser also promoted cavitation and bubble formation as part of the effluent recycling process through the fluidised bran bed in the bioreactor.

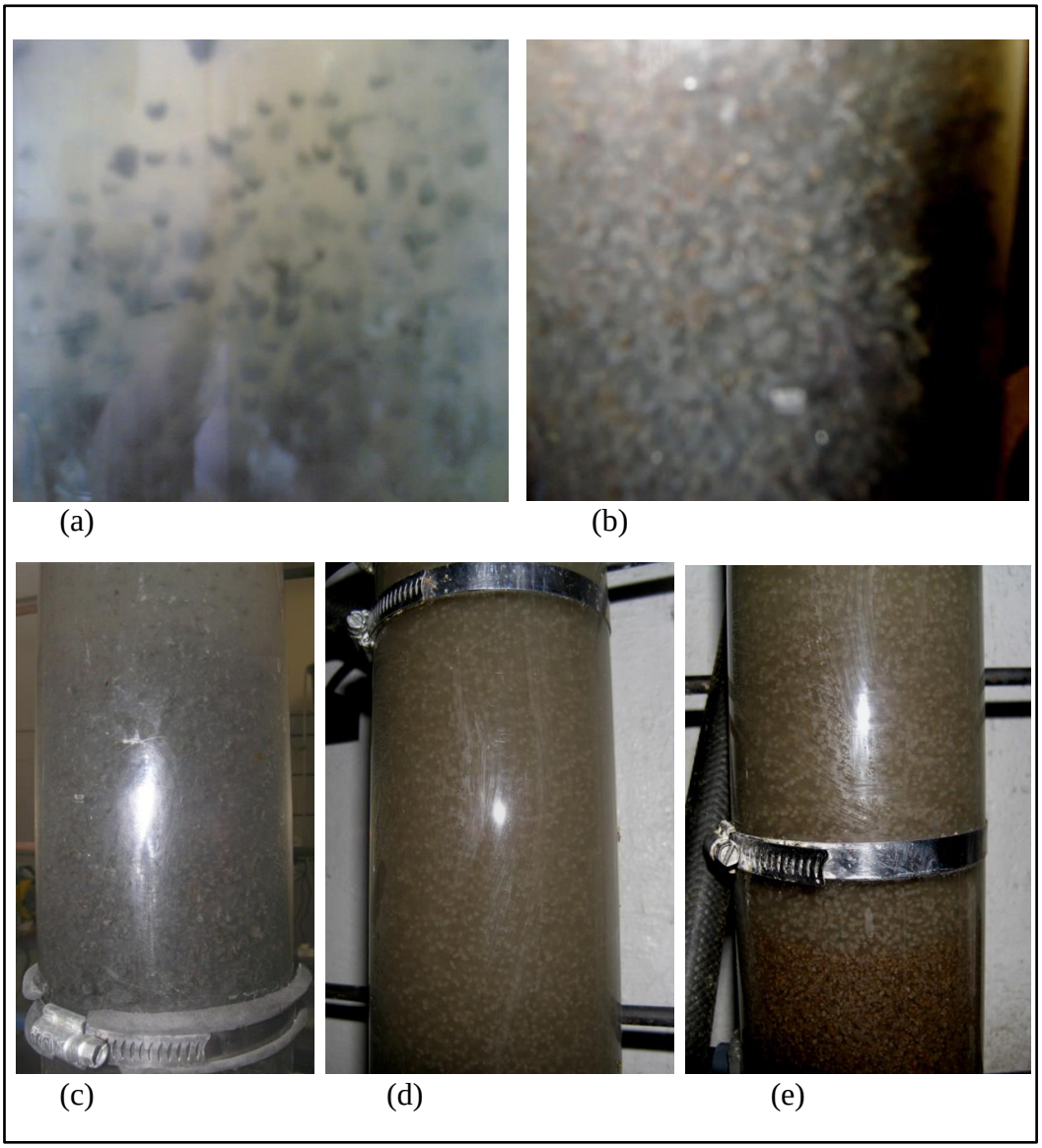


Figure 3.10: Close-up of granules in the bioreactor.

(a) Top of mesophilic floc bed clearly showing individual granules (b) Middle of mesophilic floc. (c) The base of the mesophilic granule bed. (d) and (e) Granules formed in Thermophilic bioreactor.

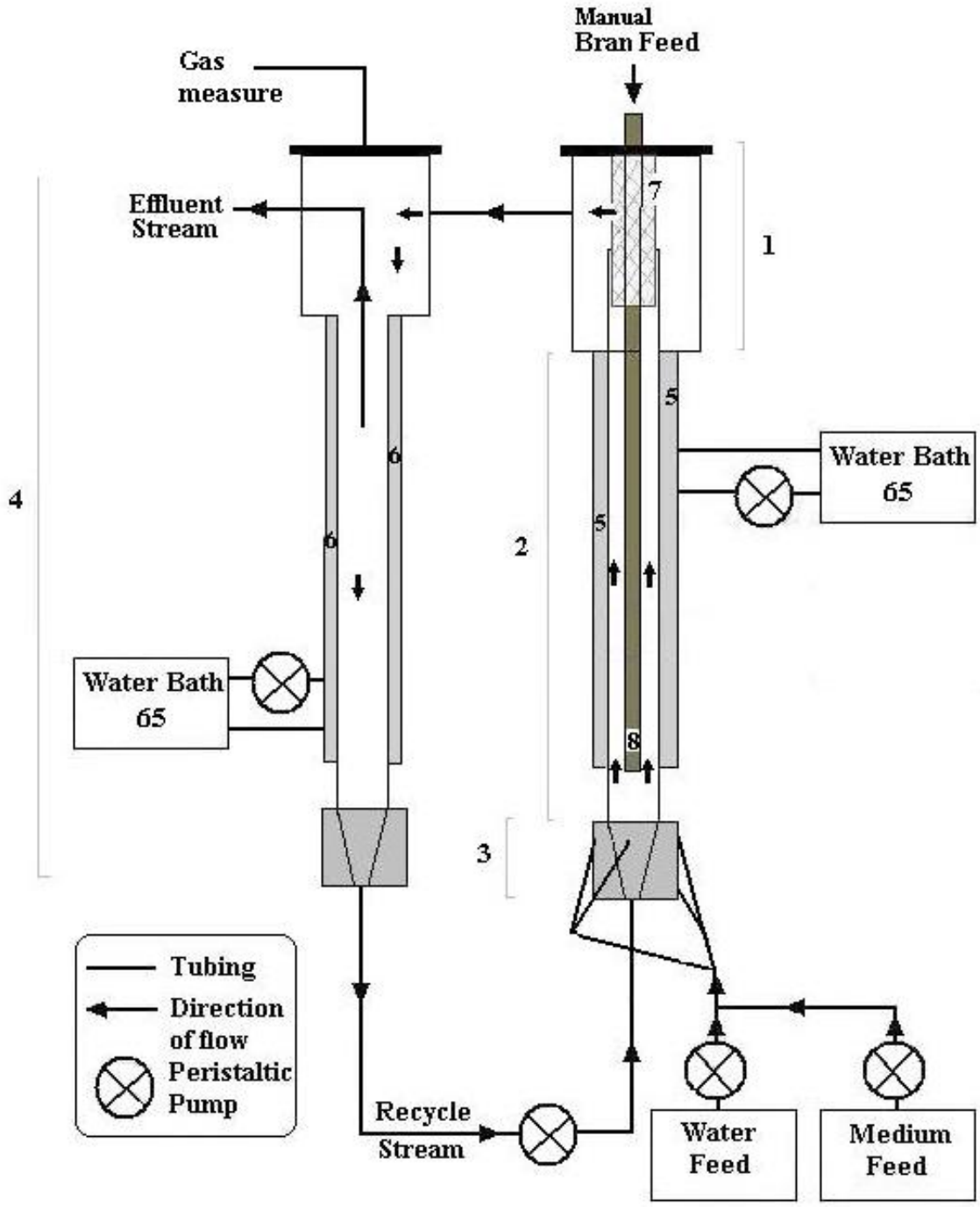


Figure 3.11: Schematic Diagram of Thermophilic Bioreactor

1-Solids-Liquids Separator, 2-Fluidised bed, 3-Feed inlet, 4-Gas Exchanger, 5-Bioreactor Water Jacket, 6-Gas Exchanger water jacket, 7-Wire Netting Cylinder, 8-Bran Feed Tube.

(e) Bioreactor Start-up

The bioreactor, consisting of the bioreactor vessel, before the gas engager attachment, was run only with the liquid medium for several days until the system operated continuously without any leakages or problems. Only after the bioreactor was running optimally on only liquid medium, was the solid feedstock added.

The bioreactor was started up by inoculating 20ml of the mesophilic consolidated consortium isolated in Chapter 2 into 6L of medium and bran at a concentration of 5g/L into the reservoir and left overnight. The resazurin indicator in the medium was light purple (aerobic) when it was inoculated but was no longer evident by the next morning, which indicated that it had become anaerobic. The following morning 5L more medium and a very concentrated mixture of bran was pumped into the reactor until the full 60cm height of the reactor was filled with suspended bran and able to circulate the medium. The new medium became anaerobic in 15 seconds as it was circulated through the reactor. The reactor was then left in batch mode to allow for an increase in bacterial biomass. The pump was set at a speed that only just kept the bran in suspension. The settled bed height of the bran initially added was 2cm.

After the maximum bran bed height was achieved, the bioreactor system was run on fed-batch while keeping the bran in suspension.

(f) Bacterial Granule Induction

Below the glass bead bed in the diffuser, a layer of cylindrical activated carbon (CAC) with a diameter of 2.5mm and length of 5.0 mm was used to promote bacterial granulation induction in the bioreactor (Lee *et al.*, 2007). Before its use, the CAC was washed in distilled water to remove all the fine particles and autoclaved for 20 minutes to ensure that it was sterile. CAC provided a settled bed height of 100mm in the bioreactor base. Nutrient medium (3.0 L) and the mesophilic bacterial consortium selected in Chapter 2 (20ml) or 1 litre of animal manure inoculum for the thermophilic reactor was added to the bioreactor systems.

3.2.2 Thermophilic Bioreactor

(a) Substrate Pre-treatment

Wheat bran was pre-treated by liquid hot water (Mosier *et al.*, 2005; Lynd *et al.*, 2002). Wheat bran was autoclaved in 1½ L of medium for the standard 20 minutes at 121 psi before being diluted to 5g/L with the medium.

(b) Nutrient Medium

Endo medium (APPENDIX A) was used to run the bioreactor. Regular tap water was used without being autoclaved. Resazurin was used to indicate when the bioreactor was aerobic and anaerobic, but no reducing agent was added.

(d) Bioreactor Setup

The thermophilic reactor system was very similar to the mesophilic set up with a few improvements and modifications to improve the efficiency of the system and prevent the problem of overflow experienced with the mesophilic system. The reactor [2] and the gas exchanger [4] were each connected to a water bath that maintained the temperature at 65°C and prevents heat loss.

As in the mesophilic reactor, the medium flowed up the reactor. Granules and bran were retained by the solid/liquid separator as well as the cylindrical metal sieve [7]. Gas was also measured from an outlet at the top of the gas exchanger, the same as the mesophilic reactor.

The recycle stream leaving the gas exchanger was directly pumped into the bottom instead of the top the bioreactor, without first passing through a reservoir. The gas exchanger thus acted as the reservoir. The effluent stream leaving the system was situated at the solids-liquids separator at the same level as the feed from the

bioreactor to the gas disengager. Liquid samples were obtained from this effluent stream.

The Endo medium was made in a concentrated form and then diluted with water before entering the reactor through the four side ports at the base of the reactor. At 8am Bran slurry, in the form of a thick paste, was added the reactor, down the feeding tube [8] to the bottom of the reactor. Gas measurements were made at intervals of four hours during the day.
(30cm) was measured.

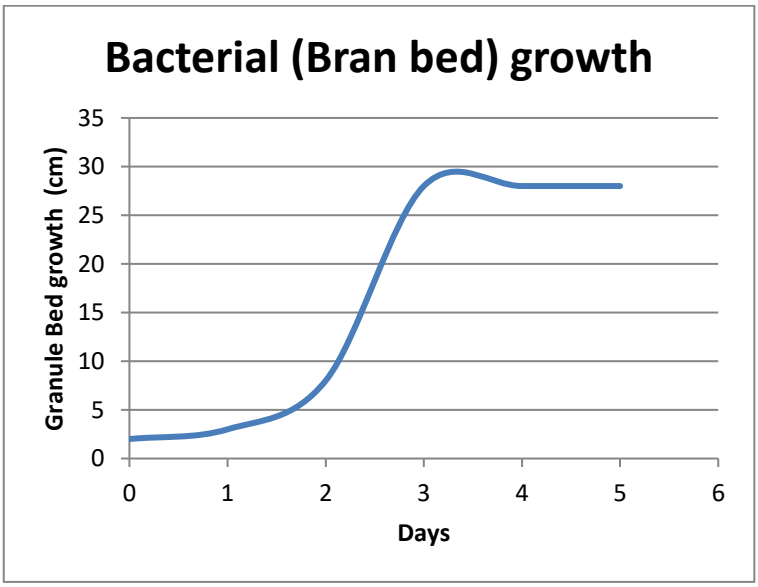


Figure 3.12: Line graph showing the rate of bacterial growth, measured as the increase in the height of the settled bran bed.

(e) Bioreactor Start-up

The reactor was inoculated then run on a batch effluent-recycle mode for 48 h at 45°C to allow the bacterial consortium to adapt to the bioreactor conditions and allow for their attachment to the CAC and bran particles. After this acclimation period, the bioreactor operation was changed over continuous effluent recycle mode with an HRT of 8h. The HRT was next gradually reduced over 48h intervals by raising the rate of nutrient medium supply. Growth and development of bacterial biofilm on the CAC/bran particles was initiated and became visible while the HRT

was decreased from 8 to 4h. As the HRT was reduced below 4h, the biofilm growth flourished inducing bacterial granules formation and accumulation on the surface of the expanded CAC/bran bed. After granule growth was initiated, further reductions in the HRT from 2 to 1.6h galvanised the swift growth and expansion of the granular bed. Figure 3.10 shows granules in the bioreactor. The HRT was continually reduced 48h intervals by raising the supply of nutrient medium to a closing rate of 4.5 L/h.

3.2.3 Analytical Techniques

(a) Determination of growth rate

The presence of planktonic as well as bacterial attachment to bran surfaces to form flocs/granules did not make it feasible to measure bacterial numbers by measuring the absorbance of the medium. The bacterial growth rate was determined by measuring the daily increase in bed height (increase in the height of floc/granule growth in the reactor).

(b) Quantification of Fermentation Products

Ethanol concentration was measured using a Quantichrome™ ethanol kit from Bioassay systems (AEC Amersham S.A). The Anthrone Method determined glucose levels. The level of H_2 produced was determined by gas chromatography.

3.3 RESULTS AND DISCUSSION

3.3.1 Mesophilic Bioreactor

After inoculation, the bacterial bran bed grew, as shown in figure 3.12. The maximum expanded bran bed height achieved, i.e., only the settled bed height

The growth rate could not be measured using simple OD because bacterial biomass was predominantly made up of flocs or granules. The increase in the bacterial bran

bed height was used as a measure of growth rate instead. Over the next three days, the bran bed grew at an exponential rate, shown in figure 3.12, with a doubling time of about a half a day. The bed height remained stable at 28cm from day three onwards, which was probably the maximum bed height that could be obtained in this reactor size, with a bran concentration of 5g/L.

Figure 3.12 shows the bran bed height growth in the reactor after inoculation, over five days of being run continuously. A very concentrated mixture of bran was pumped into the reactor until the full 60cm height of the reactor was filled with suspended bran. And the reactor was changed over to continuous feeding of bran and medium. The maximum expanded bran bed height achieved, i.e., only the settled bed height (30cm) was measured.

Anaerobic bioethanol production from lignocellulosic substrates (bran) at 65ºC in a fluidised heterogeneous bed bioreactor was demonstrated. The mixed bed was comprised of the following components: suspended bran particles, bacterial flocs, and bacterial granules.

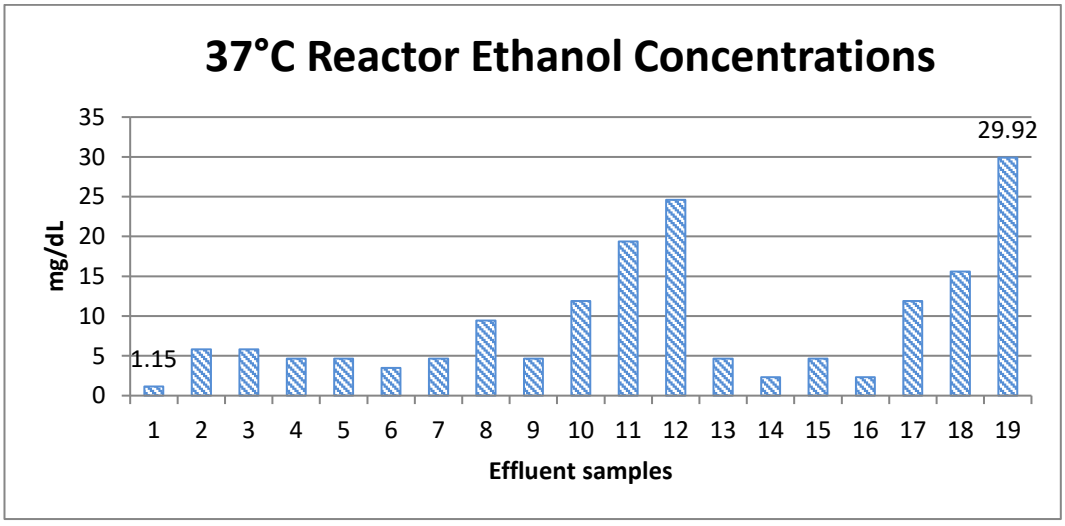


Figure 3.13: Ethanol concentrations obtained from the mesophilic reactor over 19 days

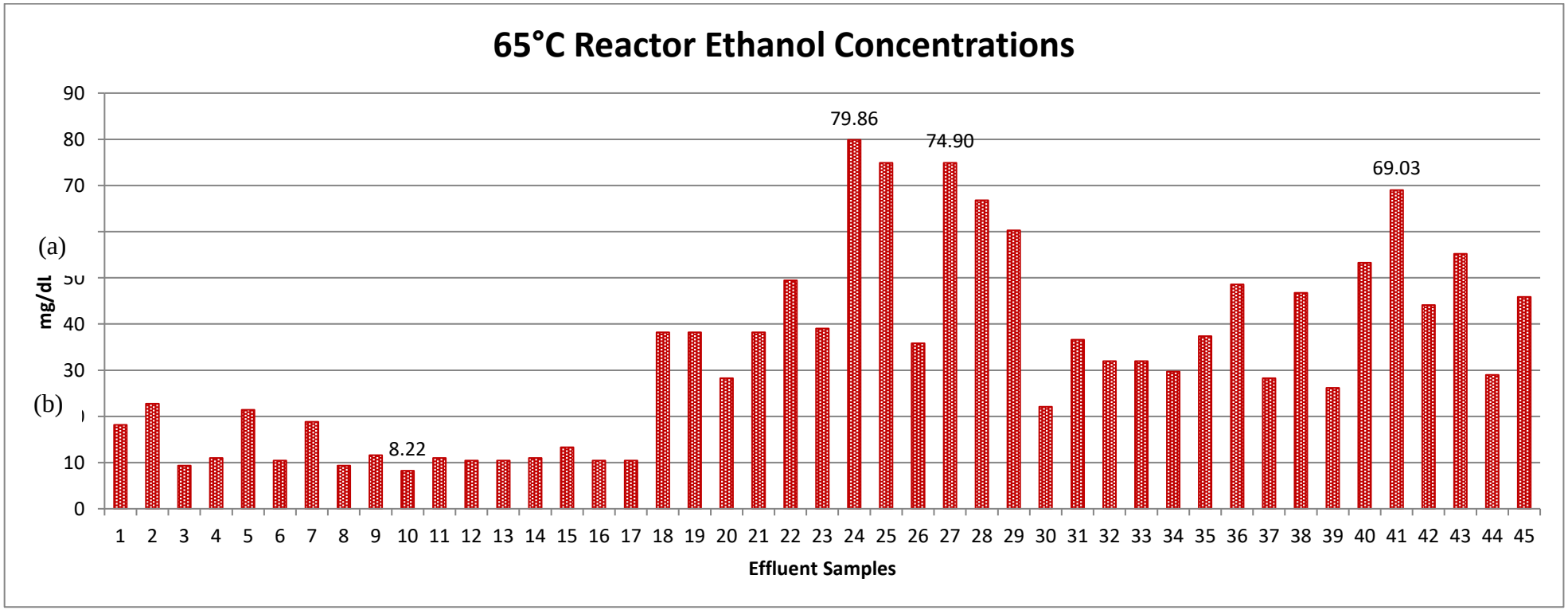
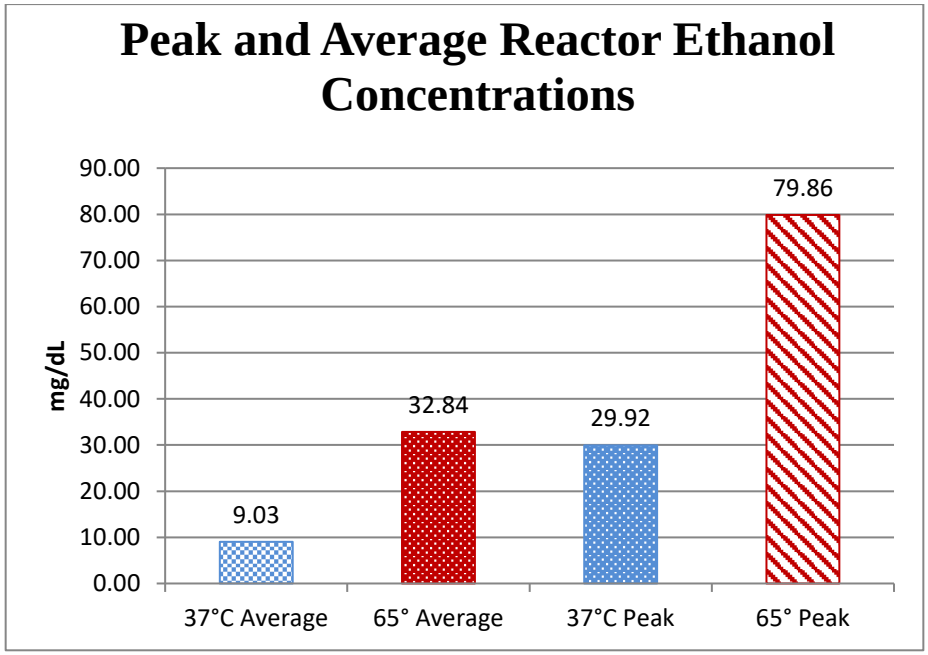
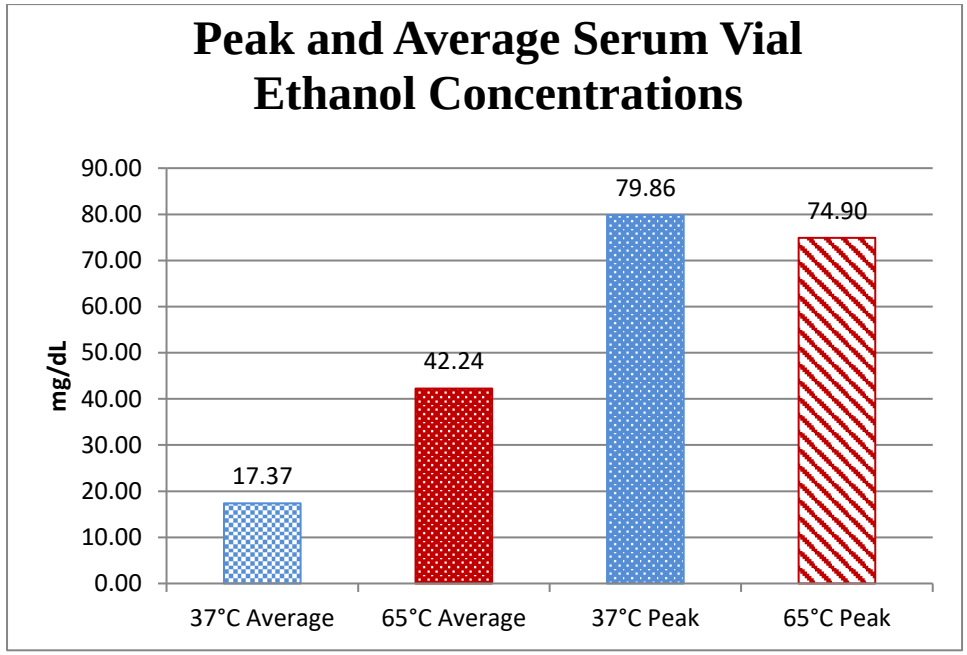


Figure 3.14: Ethanol concentrations obtained from thermophilic reactor over 45 days



(a)



(b)

Figure 3.15: Comparison of the peak and average ethanol concentrations in (a) the mesophilic and thermophilic reactors, (b) Mesophilic and thermophilic Serum Vials

In the mesophilic reactor, ethanol was measured over 19 consecutive days, Figure 3.13. During this time, reactor operation was constant interruptions due to pipe bursts, overflows and washout while trying to get the system to operate continuously. Many teething problems had to be overcome due to the novel (at the time of this research: 2008) nature of our bioreactor setup and our solid bran feedstock. The starting inoculum for this bioreactor was very small, i.e., 5ml of the consolidated consortium selected previously in Chapter 2. A consequence of the small starter culture size was that it took a long time to bulk up the bioreactor biomass and to then initiate granule formation. The slower mesophilic growth rate may also have contributed to this slow start-up time

3.2.2 Thermophilic Bioreactor

Ethanol concentration in the thermophilic reactor, Figure 3.14, was measured for 45 days with fewer interruptions than the mesophilic reactor. The thermophilic reactor was run for 45 consecutive days. At this stage, the system was optimised so that it functioned more efficiently and continuously than the mesophilic reactor. The thermophilic reactor produced higher levels of ethanol than the mesophilic reactor. The peak ethanol concentration was 79.86 mg/dL, and after 18 days, once the system was running more smoothly, ethanol values remained more than 20 mg/dL.

The peak ethanol concentrations were 29.92 mg/dL for the mesophilic reactor, and the peak of the thermophilic reactor was more than double, i.e., 79.86 mg/dL. More significantly, the thermophilic ethanol concentration was more than three times that obtained in the mesophilic reactor.

In the serum vial isolation of consortia, the average ethanol concentrations were higher than the reactor values. The difference in the peak and average ethanol concentrations were much higher under continuous mesophilic conditions than batch (serum vial). The difference was not as significant under thermophilic conditions indicating that continuous thermophilic ethanol production is more efficient than continuous mesophilic ethanol production. It also showed that under

thermophilic conditions, batch culture is not that much more efficient than continuous production.

The Hydraulic retention time and nutrient loading rate are the critical factors affecting the rate of product formation, i.e., hydrogen and, subsequently, ethanol. As the hydraulic retention time decreases, the rate of hydrogen production increases. The rate of depletion in bran bed height also increases as the hydraulic retention time decreases.

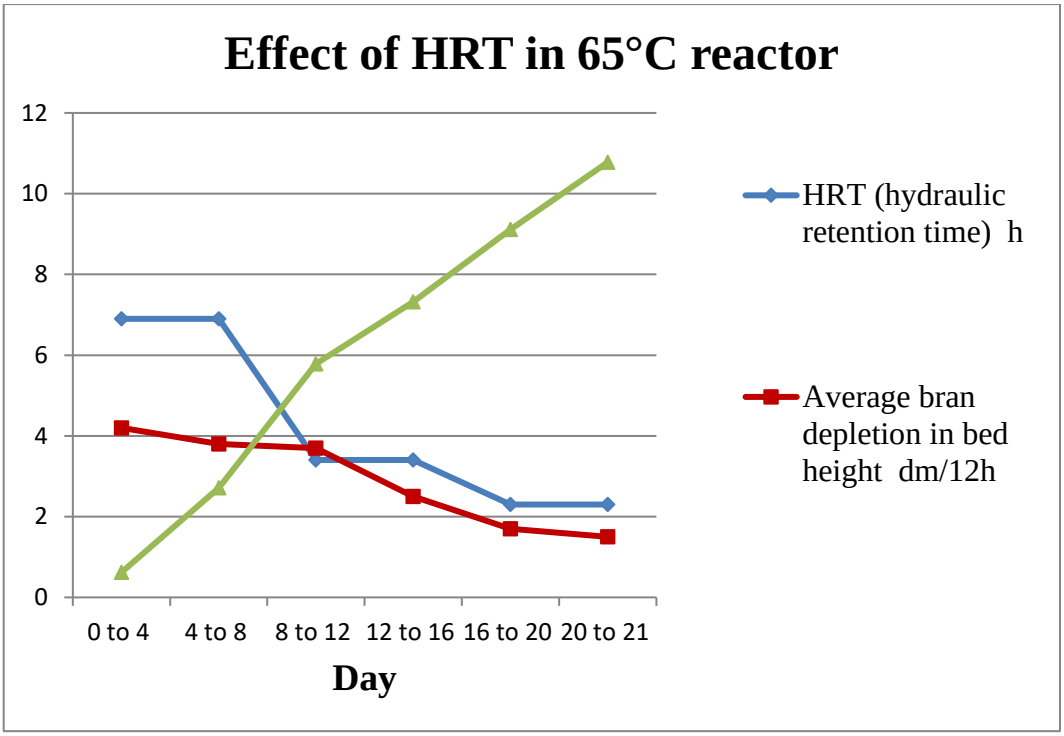


Figure 3.16: Effect of Hydraulic retention time and nutrient loading rate on substrate utilisation and hydrogen production rate.

3.3.3 Hydrogen Studies

Hydrogen and ethanol are both products of the dark fermentation of lignocellulose, and an inverse relationship exists between them. Hydrogen studies using the optimised thermophilic bioreactor and culture were performed as part of an honours project by Phumlane Masilela. The findings of this study are included below.

Table 3.1: Anaerobic biohydrogen production from a lignocellulosic substrate (bran) at 65˚C in a fluidised heterogenous bed bioreactor

Days	HRT (h)	NLR (L/h)	Effluent PH	Average bran depletion in bed height (dm/12h)	Average bran depletion in bed height (mm/12h)	Peak gas meter readings between 2 and 6pm (L/min)	Peak VHGR (L/L.h)	Peak VHPR (mmol/ L.h)	Peak hexoses in effluent (g/L)
0 to 4	6.9	0.9	8	1.5	150	0.078	0.62	21.3	0.21
4 to 8	6.9	0.9	7.88	1.7	170	0.346	2.71	93	1.63
8 to 12	3.4	1.8	5.72	2.5	250	0.736	5.78	198.3	2.17
12 to 16	3.4	1.8	6.13	3.7	370	0.931	7.32	251.1	2.98
16 to 20	2.3	2.7	5.21	3.8	380	1.16	9.11	312.5	3.6
20 to 21	2.3	2.7	5.06	4.2	420	1.373	10.78	369.8	5.32

HRT – Hydraulic Retention Time
NLR – Nutrient Loading Rate
VHGR – Volumetric Hydrogen Production Rate in L
VHPR – Volumetric Hydrogen Production Rate in mmol.

(Source: Phumlane Masilela, 2008)

At 08.00 every day, autoclaved bran was added to the bioreactor to bring the expanded bran bed height to 50 cm (equivalent to 200 g bran dry mass). Gas production was measured between 14.00 and 18 00. Anthrone analysis of sugar content in the effluent was also measured on these samples. The working volume of the fluidised bioreactor bed was 3.21L. The total capacity of the bioreactor system (bioreactor, piping, and gas-disengager) was 6.185 L. HRT = hydraulic retention time, NLR = sucrose-free Endo nutrient influent rate (pH 7.5 to 8.5), VHGR = volumetric biohydrogen gas production rate in litres, VHPR = volumetric biohydrogen production rate in mmol. Inoculum derived from rumen manure. The average biohydrogen concentration was 42 %. All gas and hexose measurements represent the average of 3 replicates.

3.4 CONCLUSIONS

The operation goal of the AFBR bioreactor system was to maintain a stably fluidised bran bed within the bioreactor. The recycling of degassed effluent through the bran bed fulfilled the following bioreactor functions: 1) fluidization of the bran bed, 2) gas removal from the liquid phase by liquid-gas phase partition in a process that was facilitated by gas bubble formation through cavitation, 3) mass transfer and mixing 4) maintenance of bacterial biomass density within the bioreactor. In summary, the bioreactor system was complex and heterogeneous in that it consisted of dynamic interactions and material exchanges between three phases: solid phase, liquid phase, and gas phase.

The solid phase consisted of the insoluble bran substrate particles and the bacterial granules, which also formed in the bed. The liquid phase consisted of the buffering media and recycled degassed effluent. The recycling of effluent was necessary for maintaining the fluidised state of the bran bed. The gas-phase comprised mainly of hydrogen.

In the bioreactor, gas was removed from the liquid phase by the process of liquid-gas phase partitioning. The interaction between the upflowing effluent and the sedimenting solid-state generated cavitation of the gas-saturated liquid phase resulted in the formation of upwardly mobile or rising gas phase in the form of gas bubbles. The decanting of the supersaturated effluent – bubble mixture from the solid-liquid separator into the gas disengager created violent turbulence within the gas disengager column resulting in the release of gas, including hydrogen. In this process, gas was stripped from the liquid phase in the gas disengager.

Optimisation of operation also required adjusting: 1) the effluent recycle rate to prevent the formation of large gas pockets in the fluidised bed which would push the entire bed upwards against the sieve of the solid-liquid separator, 2) the hydraulic retention time and, 3) the rate of batch bran feed. These were the operational challenges that were overcome, and in this sense, proof of concept was achieved about system operational stability.

Chapter 4

MOLECULAR STUDIES

4.1 INTRODUCTION

Microorganisms have traditionally been classified by characterising their morphological and physiological traits in pure defined cultures, followed by testing of multiple physiological and biochemical traits (Valinsky *et al.*, 2002). The discrepancy between plate counts and direct microscopic sampling, as well as between populations derived from culture or molecular methods, led to the belief that bacteria in natural environments may be 'uncultivable' (Ward *et al.*, 1998). This discrepancy is more likely caused by our failure to comprehend and recreate the exact micro-environmental niches that affect the bacteria occurring in nature (Muyzer and Smalla, 1998).

In liquid environments, microbes exist in a free growing planktonic phase as well as immobilised consortia on solid surfaces (Amann *et al.*, 1995). In an anaerobic fluidised bed reactor (AFBR), the aim is to get both planktonic and attached growth. A theory for the collaboration of different microorganisms into immobilised consortia or biofilms is that they often catalyse key microbial transformations that they would not be able to do individually (Amann *et al.*, 1995). It is especially true for the degradation of recalcitrant materials such as lignocellulose. There are no single bacterial species that can break down lignocellulose (cellulose) and utilise the resulting hexose and pentose sugars to produce ethanol. It is thus a better strategy to use naturally occurring bacterial consortia that function in a synergistic way to perform the whole process from beginning to end. The collaboration of bacterial consortia into biofilms provides the advantage of supplying a higher availability of nutrients and the possible long-term positioning relative to other microbes. It has only been possible recently, the use of molecular methods, to identify uncultured microbial populations (Amann *et al.*, 1995).

Ribosomal RNA (rRNA) molecules are ubiquitous to all cellular life. These molecules are made up of stretches of highly conserved sequence domains interspersed with more variable regions. In general, essential rRNA domains are conserved across all phylogenetic domains (Head *et al.*, 1998). In the 1970s, Woese and colleagues described the use of comparative rRNA analysis for phylogenetic studies. They were able to identify sequence motifs or signature sequences that provide an evolutionary basis for prokaryotic taxonomy, which has led to the three-domain organisation of the living world: Archaea, Bacteria, and Eukarya (Olsen and Woese, 1990). Short stretches of sequences characteristic of divisions, subdivisions as well as species and subspecies-specific sequences have been identified (Valinsky *et al.*, 2002).

The subsequent development of strategies to analyse rRNA molecules and rRNA genes (rDNA) finally provided a culture-independent means to examine the tremendous diversity of microorganisms inhabiting the natural world. Numerous rDNA-based strategies have been developed for mixed microbial community analysis, the most common of which are: terminal random fragment length polymorphisms (tRFLP) analysis, ribosomal intergenic spacer analysis (RISA) and denaturing gradient gel electrophoresis (DGGE) (Valinsky *et al.*, 2002).

A commonly used method is DGGE, which can be used to detect and identify microorganisms by using molecular markers for highly conserved regions of 16S rDNA (Muyzer and Smalla, 1998). During DGGE analysis PCR amplified 16S rDNA gene segments are separated in an acrylamide gel composed of denaturants with an increasing concentration gradient.

Separation in DGGE is based on the electrophoretic mobility of a partially denatured DNA molecule in polyacrylamide gels, which is decreased, compared with that of the fully helical form of the molecule. The melting of fragments follows in discrete so-called melting domains: stretches of base pairs with identical melting temperatures. When the melting domain with the lowest melting temperature reaches its melting temperature at a particular position in the DGGE gel, a transition of helical to partially

helical molecules occurs (partial denaturation), and migration of the molecule will practically halt. Double-stranded rDNA segments have differences in melting characteristics depending on sequence difference (Ward *et al.,* 1998). Sequence variation within such domains results in a difference in their melting temperature. Sequence variants of fragments of the same size will, therefore, stop moving at different positions in the denaturing gradient and hence can be separated effectively by DGGE (Muyzer *et al.*, 1992). GC-rich sequences (GC-clamp) are incorporated into one of the primers to modify the melting behaviour of the rDNA fragment of interest to the extent to which close to 100% of all possible sequence variations can be detected (Muyzer *et al.*, 1992).

Many different 16S rDNA sequences detected simultaneously in the resulting profile of bands that can each be re-amplified by PCR and then sequenced (Yang and Crowley, 2000). This procedure allows one to identify the presence and relative abundance of different species directly and thus to profile microbial populations in both a qualitative and semi-quantitative way (Muyzer *et al.*, 1992).

4.1.1 Aims

To identify and differentiate the population structure of mesophilic and thermophilic inocula and bioreactor samples and ideally to identify the bacteria that made up the bacterial consortia.

4.1.2 Objectives

The first objective was to isolate and amplify the 16S rDNA extracted from our bacterial consortia inocula and reactor samples. The second objective was to separate the consortia 16S rDNA into the different component species using the DGGE. The best protocol to achieve clear separation and clarity of the banding pattern had to be determined using different parameters such as acrylamide concentration and various

high and low urea concentrations. The third objective was to use the DGGE technique to identify individual species making up the bacterial consortia. Identification would be achieved by cutting out discrete bands, to re-amplify and send the DNA to be sequenced to identify the different bacterial species making up the bioreactor consortia.

4.2 MATERIALS AND METHODS

4.2.1 DNA Extraction

Bacterial cultures were removed from serum vials and bioreactor effluent anaerobically using a syringe before DNA isolation. The bacteria were recovered from samples by repeated microcentrifugation of the 1ml liquid sample until a significant pellet was formed. The pellet was re-suspended in sterile $_{dd}H_2O$ and used for DNA extraction. DNA was extracted using the ZR Fungal/Bacterial DNA isolation kit™ except where indicated. The ZR soil DNA extraction kit™ (Zymo research) was used according to the manufacturer's instructions as a comparison. The ZR fungal/bacterial DNA isolation kit™ was used for all subsequent extractions.

4.2.2 PCR before DGGE

Primers described by Dewettinck *et al.* (2001) based on universally conserved regions of 16S rDNA genes (Table 4.1) were enzymatically used to amplify the variable V3 region of 16S rDNA. Before DGGE analysis, the forward primer with the GC-rich clamp (40 nucleotides long) at its 5' end (Muyzer *et al.*, 1992) (P63f) was used, and the forward primer without the GC-clamp (P63F) was used for re-amplification after DGGE. The same reverse primer (P518r) was used in both cases with the expected amplified fragment length of 530 bp (base pairs). Primers were synthesised by Inqaba Biotechnical Industries (South Africa).

The PCR mixture contained: 13µl of RNAse/DNAse free water, 1µl each of both forward and reverse primers, 25µl of Mastermix (Fermentas) and 10µl of the genomic DNA extract. The total volume of 50µl was centrifuged for 5 seconds to mix thoroughly before PCR. Amplified rDNA samples were amplified using The Applied Biosystems GeneAmp® PCR System 2700 as follows: 94°C for 5 min, followed by 30 cycles of 95°C for 1 min, 53°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min (Dwettinck *et al.*, 2001)

Table 4.1: Oligonucleotide primer sequences and source of 16S rDNA templates

Primer	Sequence
Forward primer (with 60bp GC clamp) P63f	5′- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC CAG GCC TAA CAC ATG CAA GTC-3′
Forward primer (24bp without GC clamp) P63F	5′-CAG GCC TAA CAC ATG CAA GTC-3′
Reverse primer (18bp) P518r	5′-ATT ACC GCG GCT GGC TGG-3′

Source: (Muyzer *et al.*, 1992; Dewettinck *et al.*, (2001)

The presence of amplified DNA fragments was detected using the standard method of agarose gel (1.7%) electrophoresis with 0.5× TAE running buffer. PCR product (5 µl), premixed with two µl 6X Orange Loading Dye, solution (Fermentas) was loaded as well as O'GeneRuler™ 100bp DNA Ladder Plus (Fermentas). Gels were run at 100V and were then visualised and photographed using a Bio-Rad® Gel Doc™ XR Gel Documentation system.

4.2.3 Differential Gradient Gel Electrophoresis

DNA samples loaded into wells were prepared by mixing equal volumes of loading dye and (5µl) or 10µl of each PCR product. Electrophoresis was performed in a 0.5×

TAE electrophoresis buffer (20 mM Tris-HCl, pH 8.3; 10 mM acetic acid; 0.5 mM EDTA), a temperature of 63°C. Different voltage, differential gradients, and acrylamide concentrations variable were used for electrophoresis, as indicated in Table 4.2, to obtain the best banding pattern. After electrophoresis, the gel was floated off the glass plates into the staining solution, to minimise damage to the delicate DGGE gels due to handing. It was stained in 250ml running buffer and 25µl of 10 mg/ml ethidium bromide for 15 minutes, and then de-stained for 15 minutes in running buffer. After staining, gels were viewed and photographed using a BioRad® Gel Doc™ XR+ Gel Documentation System. All visible bands were carefully excised from the gel, being careful to remove all excess gel.

Table 4.2: Different DGGE variables tested

	Acryl amide	Urea Concentration Gradient	Run Time	Temp	Voltage	The volume of sample loaded into wells
(a) Gel 1	8%	45% to 65%	2.5 h	65°C	200V	5 µl
(b) Gel 2	6%	40% to 60%	3 h	65°C	200V	5 µl
(c) Gel 3	6%	20% to 50%	3 h	65°C	130V	5 µl
(d) Gel 4	6%	30% to 65%	4 h	63°C	130V	10 µl
(e) Gel 5	6%	0% to 35%	17 h	60°C	100V	10 µl
(f) Gel 6	6%	20% to 60%	17 h	60°C	100V	10 µl

For consistency, only BIO-RAD chemicals and reagents were used. The DGGE was performed using a DCode™ Universal Mutation Detection System (Bio-Rad) according to the manufacturer's instructions. This system allows for two gels to be run at the same. After the gel sandwich was assembly, the bottom of the glass plates was sealed using agarose gel to prevent leakage of gel when pouring.

High and low solutions were prepared by mixing the 100% and 0% stock solutions to the desired percentage, e.g., 9 ml of the 100% denaturing solution, and 6 ml of 0%

denaturing solution makes 15 ml of a 60% denaturing solution. A final concentration of 0.09% (v/v) each of the polymerising agents used: ammonium persulphate (135µl) and TEMED (13.5µl) was added to each high and low solutions immediately before pouring the gel. Gels of 0.5mm thickness with 17 wells were cast using the gradient former included in the Bio-Rad® system according to the manufacturer's instructions.

4.2.4 16S rDNA Extraction from Gel Slices

DNA bands were carefully cut out of gels using a flat blade and then placed into sterile Eppendorf tubes with 50µl of sterile distilled water. Various methods to extract the 16S rDNA template from the excised bands were used. Each gel slice was placed into a separate sterile Eppendorf tube. The gel slices were processed using the methods shown in Table 4.2. When using DNA extraction kits, the gel slices were left intact, but when using Method 1 and 3 Gel slices, broken up to increase the efficiency of DNA extraction. Gel slices were broken up by centrifugation in 0.6 ml microfuge tubes with a pinhole in the bottom and collected in a 1.5 ml Eppendorf tube, shown in Figure 4.1, or by pushing the gel through a syringe needle. When the gel slice was broken up, one of the following two methods were used to remove polyacrylamide pieces from the extraction medium: separation through a syringe containing a plug of glass wool or centrifugation. Microfuge tubes were centrifuged for 10 minutes at 14,000 rpm to pellet the polyacrylamide out of solution.

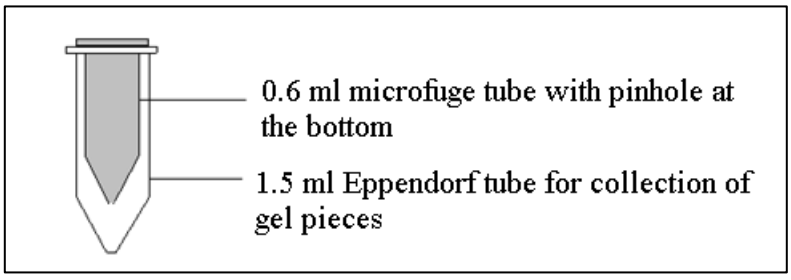


Figure 4.1: Diagram showing the assembly of microfuge tubes used to break up DGGE gel slices.

4.2.2 PCR After DGGE

Primers described by Dewettinck *et al.* (2001) based on universally conserved regions of 16S rDNA genes (Table 4.1) were enzymatically used to amplify the variable V3 region of 16S rDNA. Forward primer without the GC-clamp (P63F) was used for re-amplification after DGGE. The same reverse primer (P518r) was used in both cases with the expected amplified fragment length of 530 bp (base pairs). Primers were synthesised by Inqaba Biotechnical Industries (South Africa).

Table 4.3: Methods used to extract DNA template from gel slices after DGGE

	Method 1 Sterile $_{dd}H_20$	**Method 2** Quiex Gel DNA extraction kit	**Method 3** Crush and Soak	**Method 4** DNA clean and concentrate kit (Zymo Research)
Incubation (extraction) Medium	50 µl sterile $_{dd}H_20$	3 volumes of extraction buffer	Sufficient extraction buffer (from method 2) or $_{dd}H_20$ to cover gel slices	DNA binding buffer
Incubation Temperature	55°C	55°C	Room Temperature	Room Temperature
Incubation Time	overnight	30 minutes	Overnight	2 minutes
Eluate used for PCR	Water	Purified DNA	Purified DNA	Purified DNA

The PCR mixture contained: 13µl of RNAse/DNAse free water, 1µl each of both forward and reverse primers, 25 µl of Mastermix (Fermentas) and 10µl of the genomic DNA extract. The total volume of 50 µl was centrifuged for 5 seconds to mix thoroughly before PCR. Amplified rDNA samples were amplified using The Applied Biosystems GeneAmp® PCR System (Bio-Rad) 2700 as follows: 94°C for 5 min, followed by 30 cycles of 95°C for 1 min, 53°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min (Dwettinck *et al.*, 2001)

Method 1: Sterile ddH20

Polyacrylamide gel slices were placed into 50 µl sterile $_{dd}H_20$ and incubated at 55°C overnight

Method 2: Quiex Gel DNA extraction kit

Polyacrylamide gel slices were placed into 3 volumes of extraction buffer and incubated at 55°C for 30 minutes

Method 3: Crush and Soak Protocol

A hot needle was used to make a hole in the bottom of a 0.5ml microcentrifuge tube. The gel slice is placed into a microfuge tube and, in turn, into a 1.5ml tube. The assembly is centrifuged at top speed (14k rpm) for 1-5 minutes, or until the entire gel slice is collected in the lower tube.

1. Sufficient buffer was added to the gel pieces to cover them.
2. This slurry was incubated at 37° C with shaking for 2-12 hours. Longer incubations give better recoveries and are essential for the acceptable recovery of fragments larger than 500bp.
3. The microfuge tube was spun for 10 minutes at 14,000 rpm.
4. The eluate was removed to a 1.5 ml microcentrifuge tube
5. Another 500 µl of crush and soak solution was then added
6. The spin was repeated, and the recovered supernatant was pooled.
7. Polyacrylamide fragments were carried over into the supernatant and recovered by a second centrifugation step.
8. Glycogen (10 µg) was added and 2.5 volumes of 100% Ethanol.
9. The samples were chilled at -20°C for approximately 30 min.
10. The samples were centrifuged for 5 min at maximum speed in a microcentrifuge to pellet the DNA.
11. The supernatant was discarded and resuspend the DNA pellet in 200 µl of TE Buffer.

12. Sodium Acetate (25 l of 3M) and 750 µl of 100% ethanol was added. Steps #9 and #10 were then repeated
13. The supernatant was discarded and resuspended the DNA pellet in 80 µl TE Buffer, using two 40 µl aliquots.

Method 4: Quiex Gel DNA extraction kit

Polyacrylamide gel slices were placed into DNA binding buffer and incubated at room temperature for 2 minutes

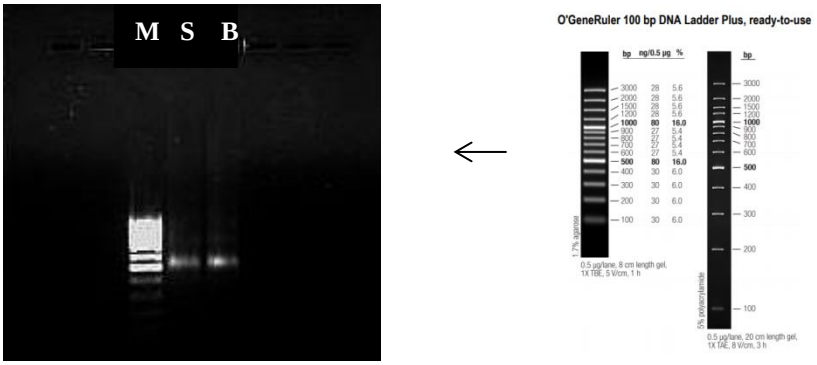
4.3 RESULTS AND DISCUSSION

4.3.1 16S rDNA Extraction

Both the ZR Fungal/Bacterial DNA isolation kit™ and the ZR soil DNA extraction kit™ (Zymo research) worked well for the isolation of 16S rDNA gene segments from our bacterial consortia. Figure 4.3 shows the PCR products of the two kits, both producing good bands and produced equally intense PCR bands.

4.3.2 Polymerase Chain Reaction (PCR) before DGGE

A template with the size of ±530bp amplified by PCR was confirmed for all cultures before being used for DGGE. Figure 4.2 is a typical example of an agarose gel showing the presence of 16S rDNA sequences from the bacterial consortium obtained from the mesophilic bioreactor.



(a) (b)

Figure 4.2: Agarose Gel showing PCR products before DGGE

(a) Agarose gel of two isolation kits: M - 530pb molecular marker S - ZR soil DNA extraction kit™. (Zymo research), B - ZR fungal/bacterial DNA isolation kit™ (Zymo research) except where indicated. (b) O'GeneRuler™ Gene ladder used.

4.3.3 Differential Gradient Gel Electrophoresis

(a) Parameters for Optimisation of Banding Pattern

The DGGE apparatus allows two gels to be run in parallel, but it was found early on that the back gel which is closer to the heating mechanism did not produce good band separation (results not shown); therefore only the front gel was used for all results shown in table 4.3. Many gels were run during the process of learning the DGGE system before good band separation was observed.

Table 4.4 shows the progression of the leading fronts of bands, with better separation of bands. The bands on gel 5 travelled more than half the length of the gel. When using a urea concentration of 45% low, the rDNA strands did not move far enough along the gel to differentiate into individual bands. The distance increased as the low urea concentration decreased to 20%. 0% did not work as well as 20%. The difference in the high and low urea concentrations was also significant. As the gap increased from 20% to 40 %, the bands travelled further down the gel. The differential gradient of

20% and 60% urea concentration allowed the bands to pass down the DGGE gel the furthest.

Table 4.4: DGGE images of Gels 1 - 6 with Gel lanes showing the furthest distance travelled in each gel

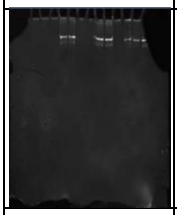
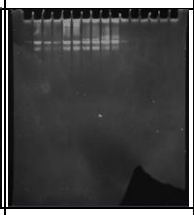
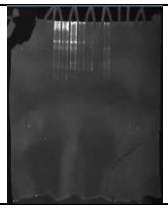
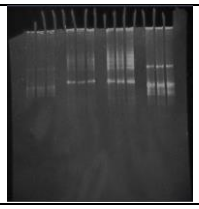
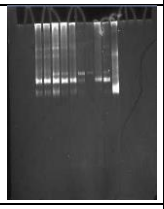
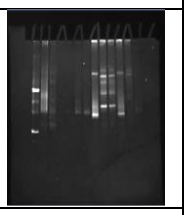
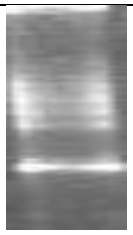
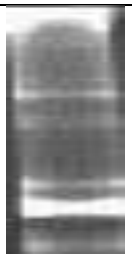
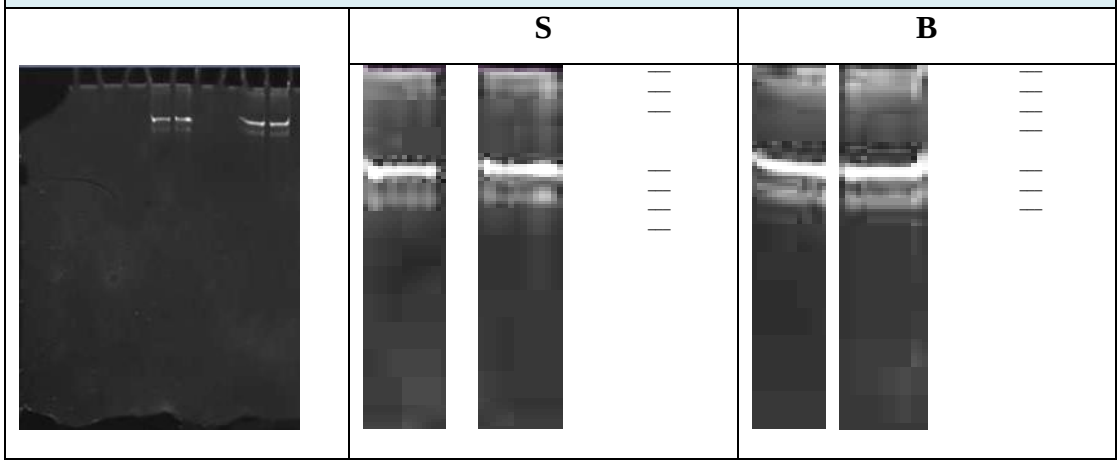
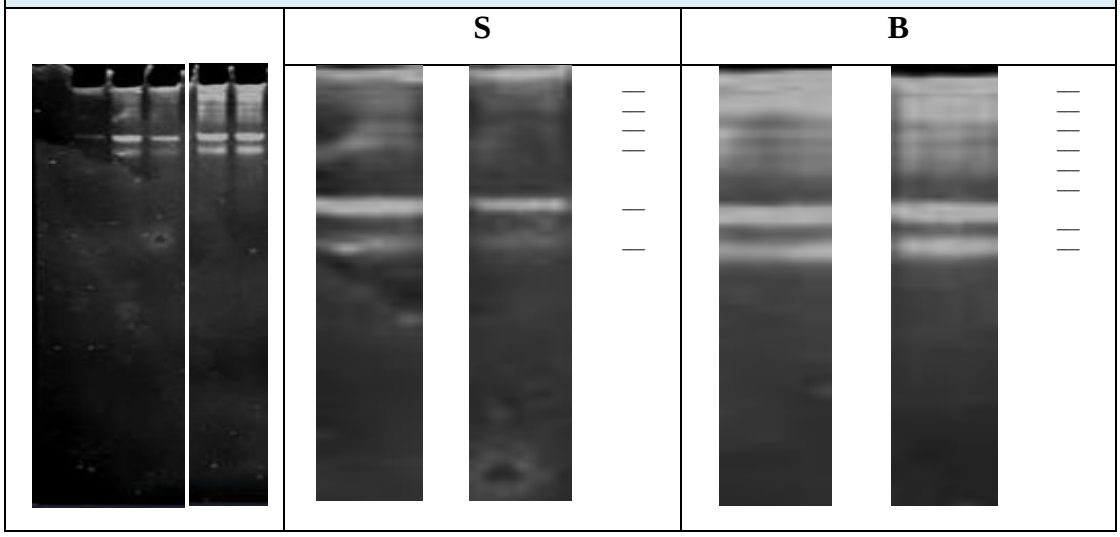
Gel 1	Gel 2	Gel 3	Gel 4	Gel 5	Gel 6
					
8% Acryl 45-65% Urea 2.5 h 65°C 200V 5 µl	6% Acryl 40-60% Urea 3 h 65°C 200V 5 µl	6% Acryl 20-50% Urea 3 h 65°C 130V 5 µl	6% Acryl 30-65% Urea 4 h 63°C 130V 10 µl	6% Acryl 0-35% Urea 17 h 60°C 100V 10 µl	6% Acryl 20-60% Urea 17 h 60°C 100V 10 µl
 **15.2 %**	 **16.6 %**	 **28.9 %**	 **38.9 %**	 **43.3 %**	 **57.9 %**

Table 4.5: Gel 1 - 8% Acrylamide, 45-65% Urea concentrations (20% difference)



Initially, the manufacturer's instructions were followed, after which different variables were used to get more distinct separating of bands. The various gels shown in Table 4.3 broadly shows the different banding patterns produced when using different high and low urea concentrations. Before testing different urea concentrations, though we determined that an acrylamide concentration of 8% (Gel 1) did not produce as good band separation as was observed in subsequent Gels (Gels 2-6) with an acrylamide concentration of 6%. We concluded that 6% was the best acrylamide concentration for our sample and setup.

Table 4.6: Gel 2 - 6% Acrylamide, 40-60% Urea concentrations (20% difference)



Run time was also found to be an essential factor with longer run times extended the leading edge of the bands. A longer run time gives the DNA segments as much time as they need to travel in the gel and get more apparent separation. A run time of 4 hours (Gel 4) produced a better banding pattern than the previous Gels 1-3. A maximum run time of 17 hours produced clearer and more distinct bands than the preceding four gels. The maximum of 17 hours was concluded to be the optimal run time to generate unambiguous and most distinct bands.

The bands also didn't travel much on the gel. A lower percentage of acrylamide of 6%, with urea concentrations of 40% and 60%, (Fantroussi *et al.*, 1999) was used for Gel 2 to allow the rDNA to move further down the gel and to get greater separation of bands. The lower acrylamide percentage successfully increased the distance that the rDNA fragments travelled down the gel, producing a better separation of bands. Even though more discrete bands could be seen, they were still too close together. In Gel 3, a lower high urea percentage of 50% with a more substantial difference of 30% instead of 20% used in Gel 1 and 2, extended the leading edge of bands even further.

Table 4.7: Gel 3 - 6% Acrylamide, 20-50% Urea concentrations (30% difference)

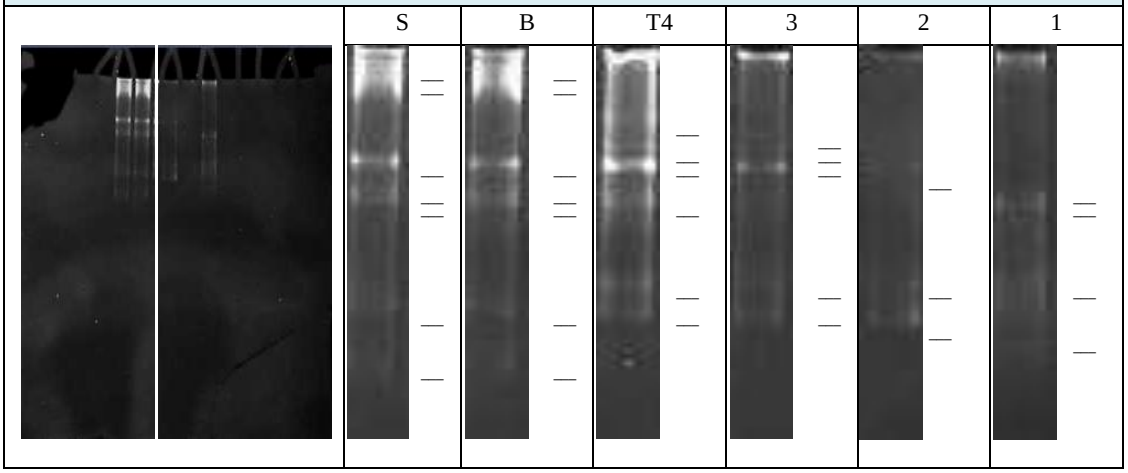


Table 4.8: Bacterial consortia samples used for DGGE analysis

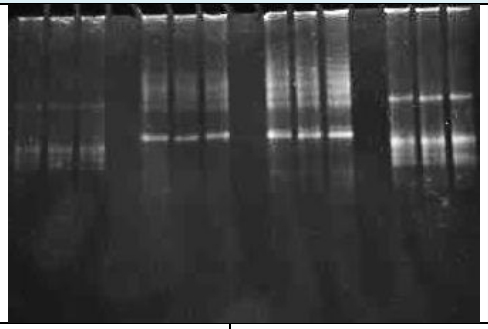
T1	Termite Culture - 65°C Original
T2	Termite Culture - 65°C sub
T3	Termite Culture - 37°C Original
T4	Termite Culture - 37°C sub
T5	Termite Culture - 37°C sub 2
T6	Termite Culture - 65°C final
T7	Termite Culture - 37°C final
S	Bioreactor Sample - Soil DNA extraction kit 37°C
B	Bioreactor Sample - Fungal/Bacterial DNA extraction kit 37°C
1	Bioreactor Inoculum - 37° C
2	Bioreactor Inoculum - 65° C
3	Bioreactor Sample - 37°C
4	Bioreactor Sample - 65°C
Cl	*Clostridium thermocellum*

Bands were further apart but feint and not very well defined. The bands higher up on the gel did not show good separation. In Gel 4 the most extreme low urea percentages of 0% and 35% allowed the bands to move even further down the gel, but there was not very good separation of bands, especially along the leading edge. The very bright band toward the leading front of the bands is most likely a few bands that have not separated. The rest of the bands were faint and not well defined.

Gel 1, Table 4.5, had two very bright and distinct bands that moved a very short distance were very close to the loading wells. These two bands were excised but were not successfully eluted from the gel slices using Method 1 in Table 4.3. There were a few very faint bands that could not be identified with the naked eye. There was a notable similarity in the banding pattern that showed that both DNA extraction kits worked equally well.

In Gel 2, Table 4.6, The two very distinct bands were present in this gel as well except that they travelled a bit further in the gel. Besides the two very bright bands, the other bands were more distinct and separated. B – (Fungal/Bacterial DNA extraction kit 37°C) produced a more evident banding pattern than S - (Soil DNA extraction kit 37°C).

Table 4.9: Gel 4 - 6% Acrylamide, 30-65% Urea concentrations (35% difference)



Universal Primers		Normal Primers	
S	B	S	B

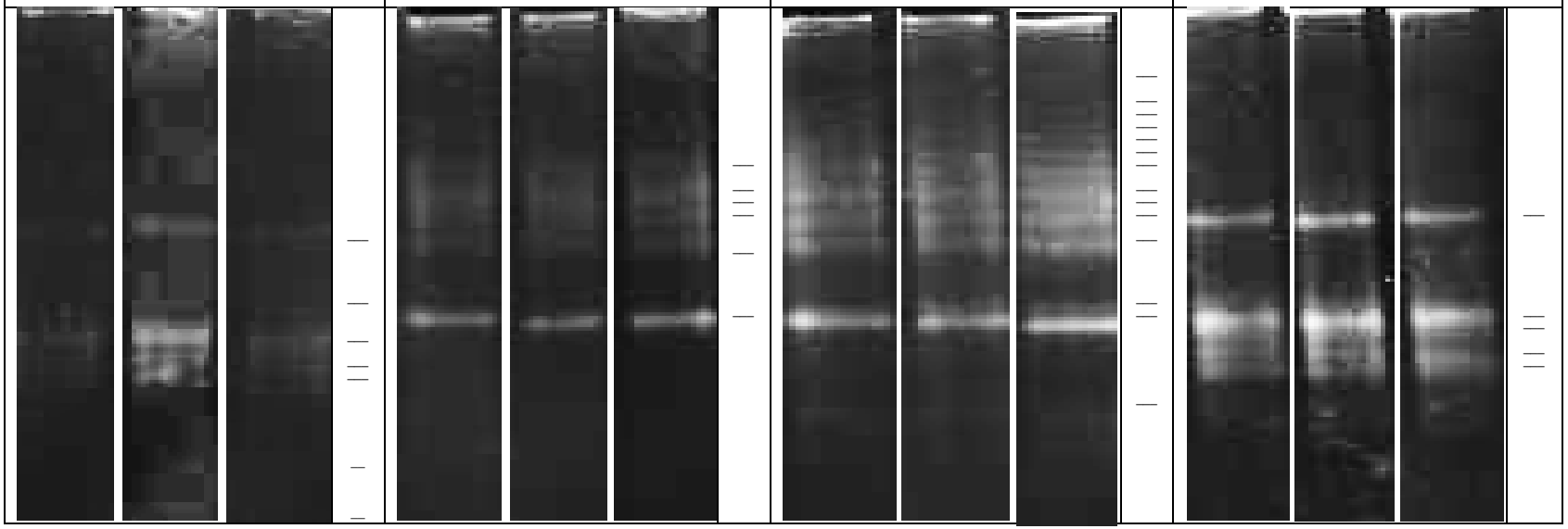
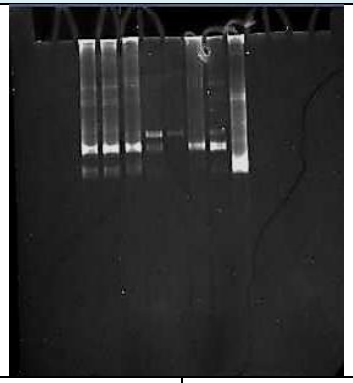
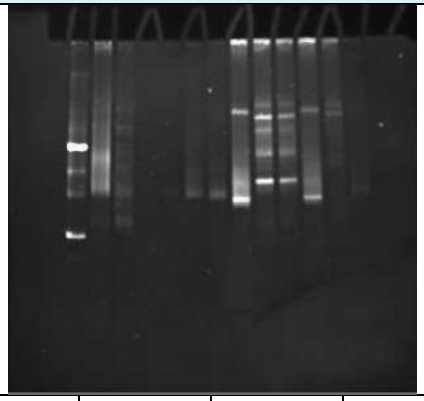


Table 4.10: Gel 5 - 6% Acrylamide, 0-35% Urea concentrations (35% difference)



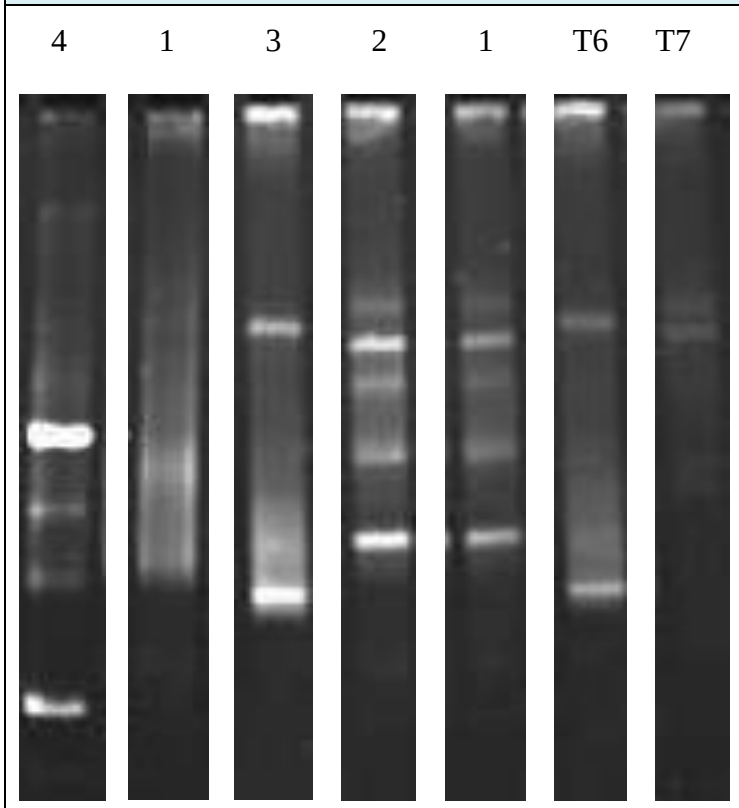
S	B	3	2	1	T5	T4	T2

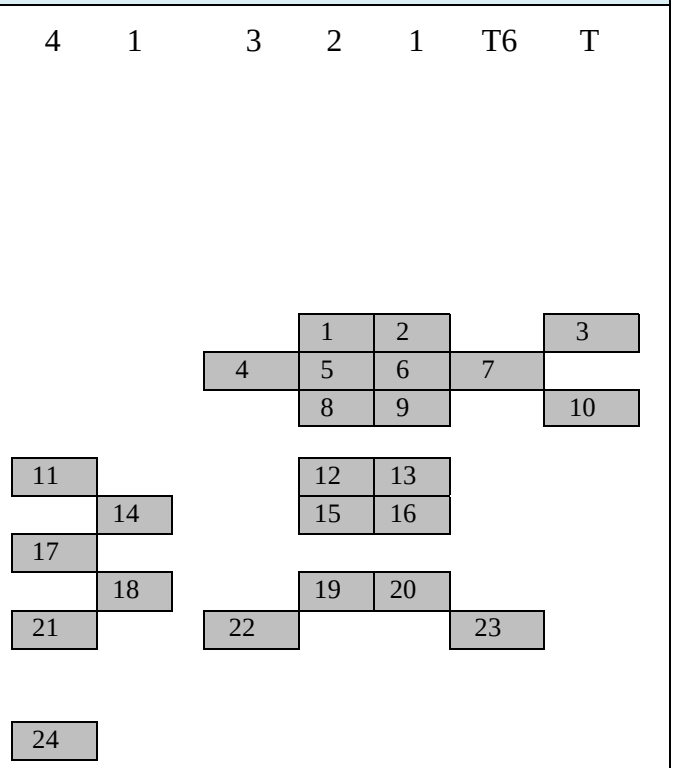
Table 4.11: Gel 6 - 6% Acrylamide, 20-60% Urea concentrations (40% difference)



3	1	2	T1	T2	T3	T4	4	4	T6	T7	Cl

Table 4.12: DNA bands excised from DGGE Gel 6, using extraction Method 4





In Gel 3, Table 4.7, The bands travelled further down the gel than Gel 2, and there was a better separation of the bands further down the gel. S and B again had the same banding pattern, but there ware still many bands higher up in the gel that was not separated well enough for excision. The bands in samples 1, 2 and 3 were much too faint for excision.

In Gel 4, Table 4.9, the bands again travelled further down the gel to get brighter bands 10 μl of PCR sample was loaded into DGGE wells of Gels 4, 5 and 6 instead of using the standard 5 μl as was used in Gels 1, 2 and 3. The higher sample volume succeeded in producing more intense bands. This gel shows the DGGE results of the same sample, a 37°C reactor sample using two different primers and 16S rDNA extraction kits. Each sample was run in triplicate to provide three samples of each separated band. Within each triplicate, some bands were more distinct than their counterparts. It is better to run samples in duplicate or triplicate to increase the chance of getting bands containing a single species.

Gel 5, Table 4.10, did not have a remarkable separation of bands and bands were not distinct. Between bands were rDNA connecting bands. Clearly defined bands are necessary to ensure that only a single species is excised in a gel slice. The Intense band in T2 is likely to include more than one species that did not separate into distinct bands.

Gel 6, shown in Table 4.11, shows the best separation and most distinct banding pattern. This Gel produced the most intense individual bands that were easy to see and cut out.

The leading front of bands on DGGE gels extended further and down the gels as we progress from Gel 1 to Gel 5, with Gel 5 showing the most significant distance of more than halfway down the gel. Although this was the best result we obtained, it could still be improved. Ideally, the leading front of bands should extend to the end of the DGGE Gel. Further adjustment and refinement of the urea concentration gradient would be

required. Alternatively, a perpendicular gradient gel could be run to determine the ideal urea concentration gradient.

Table 4.13: Methods used for extraction of 16S rDNA from DGGE gel slices

	Method 1 Sterile $_{dd}H_20$	**Method 2** Quiex Gel DNA extraction kit (Quiegen)	**Method 3** Crush and Soak	**Method 4** DNA clean and concentrate kit (Zymo Research)
Incubation (extraction) Medium	Gel slices covered in 50 µl sterile $_{dd}H_20$	3 volumes of extraction buffer or $_{dd}H_20$	Sufficient extraction buffer (from method 2) or $_{dd}H_20$ to cover gel slices	
Incubation Temperature	55°C	55°C	Room Temperature	Room Temperature
Incubation Time	overnight	30 minutes	Overnight	
Eluate used for PCR and sequencing	DNA in $_{dd}H_20$	Purified DNA	Purified DNA	Purified DNA

The 37°C and 65°C inocula have a very similar banding pattern with similar bands appearing at the same levels. Bands on the same level of the DGGE gel are the same species. Both 37 and 65°C samples were derived from the same soil and termite cultures. After selection in serum vials, the consortia isolated were still made up of the same species that could operate at mesophilic temperatures.

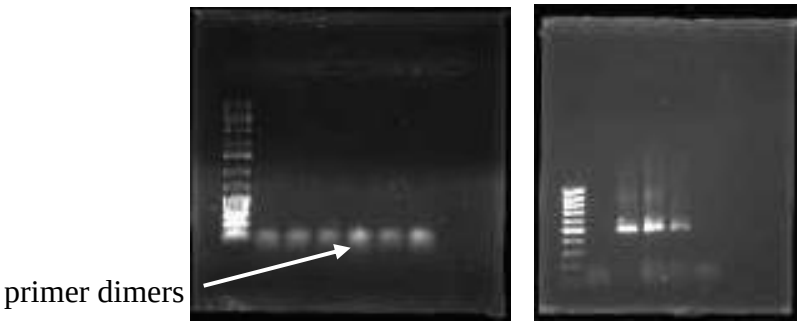
It was interesting to note that the 37 and 65°C inocula show a similar banding pattern indicating that they are both composed of comparable species that can be selected to operate at either mesophilic or thermophilic temperatures. Even though the source of all both bacteria consortia was mesophilic, the mesophilic, the same mesophilic species could be adapted to operate at thermophilic temperatures as well. The species making up the consortia were the ones responsible for the best degradation of filter paper.

4.3.4 16S rDNA Extraction from DGGE Gel Slices

Four methods were tested to extract DNA from the bands sliced out of the DGGE run on polyacrylamide gels. Extraction methods 1, 2 and 3 were not successful in extracting rDNA from the polyacrylamide gel slices of the bands visualized on the DGGE gels. Extraction method 4, using the DNA Clean and Concentrate Kit (Zymo Research) worked well. Table 4.12 shows the bands that were excised from the DGGE gel and the DNA extracted. PCR confirmation of ±600bp rDNA segments from all of the DGGE bands extracted are not shown.

4.3.5 Polymerase Chain Reaction after DGGE

Confirmation was done by PCR using primers described by Dewettinck *et al.* (2001) based on universally conserved regions of 16S rDNA genes (Table 4.1) were used for re-amplification of rDNA eluted from DGGE gel slices. The forward primer without the GC-clamp (P63F) (Muyzer *et al.*, 1992) was used for re-amplification after DGGE. The same reverse primer (P518r) was used as was used in the PCR before DGGE.



(a) (b)

Figure 4.3: Typical PCR gel showing (a) a negative result and (b) confirmation of the presence of ±530bp 16S rDNA fragments.

4.3.6 16S rDNA Sequences Obtained

Of all the samples sent for sequencing to Inqaba Biotech, only two sequences, shown below, were obtained. Sequence 1 was obtained from isolate 11, and sequence 2 was obtained from isolate 22 (Table 4.12). The sequences obtained could not be used to identify bacterial species. There may have been contamination or degradation rDNA of the samples in transit.

Sequence 1

cgcsscwcggggcgktgtcggscrgsyttycgggsgsyrmrgstkracctyywmccy
kgwaramgggrawaccmcmccraaggggksytawtcccgrawawkgycaarasssg
smttcktttttcaaaaaargrrggrawyccyttwtrgrrgrayycsscsyccccttwr
sytrtskgkkrrggkwamsgsccwmccarggsrmraakggkwrscsgyctkgarggg
ywaaagggycmcmttgaamytgaramscggycwrawctcttmsggrrgsmrcmkkkg
gwaawtttkgkgmawgggggrasmccykrmcccrscmmccccsskkrkwkaaaaarg
gcyttsggkykrwaarsytmawwkktkkggaaaaaaaaawkgmsgkwccmttmmaa
asscccsgsytayytmskkgcmasccrscsssgkatatk

Sequence 2

gcgstwssttkgttwkatttascgscgrmsggkkrastamcmskkggktamcykscy
ymwwarrgggrawtmccyycccraarggrakwtwawwmccsmawawkgwwkraaawy
csywkrwtttttkwmcmaargrasmawyccyytwtraaakgrmcccssgscccwtta
kytktttggkraggwaarggytccccarggcsacawtsckwacccamcytrarrggg
krwycgsccmmttkgaamytraaacmsgkycmaaayycywmsggrrggmrsmrkkgg
grawtwtkgcsmakgggggraamccymrmcrcmccmccccccsskkrawkraaaargg
cytwrggkttkwaarkttytktcwtwkggaaaaatawkrmsgkmmcmwtwtragraa
cccmsggywamytmckksccscccrccccssgwwataaaaa

4.3.7 Challenges

Finding the best urea concentrations was tricky because there are so many variations in High and Low concentrations that are possible. Gel 1 using 8% acrylamide with The polyacrylamide gels were made with a denaturing gradient ranging from 45% to

65% urea concentrations, with a run time of 3 hours, produced only two significant bands and a few very faint ones.

4.4 CONCLUSIONS

The different DGGE variables that were tested were: acrylamide concentration, urea concentration, run time, run temperature, voltage and volume of sample loaded into wells. The 6% acrylamide produced a better separation of bands. The maximum 17 hours run as opposed to the 2.5-hour standard, was found to create a much sharper banding pattern. The increased run time made it necessary to decrease the temperature from 65°C to 60°C and a lower voltage from 200V to 100V. Another essential factor that made a big difference to the clarity and intensity of individual bands was using 10µl instead of the standard 5µl of DNA sample into DGGE wells.

The high and low urea concentrations were optimised. The combination of 20% Low and 60% high urea concentration provided the best separation and the clearest bands.

The DNA clean and concentrate kit from Zymo Research was the only method of the four used that worked successfully to extract the rDNA from Gel slices, as confirmed by PCR. It is not clear why we were not able to receive viable sequences from these results. There may have been contaminated or not a single species present in the rDNA amplified and sent for sequencing. Even though we did not manage to identify any bacterial species that were present, it was clear that it was a mixed consortium of different bacterial species. Bands on the same level within the same gel indicted the same species, and there were commonalities among the consortia.

Chapter 5

GENERAL CONCLUSION, DISCUSSION AND RECOMMENDATIONS

A microaerophilic culture method was adapted to refrain from using a reducing agent. This method uses anaerobic methods but allows trace amounts of oxygen in serum vials. Strictly anaerobic spore formers are not able to sporulate in the presence of oxygen. Facultative anaerobes can metabolise in aerobic and anaerobic environments. They removed the residual oxygen creating an anaerobic environment required before strict anaerobic spores can sporulate. A microaerophilic culture method is thus ideally suited to isolating a mixed culture containing strict anaerobes and facultative anaerobes.

Winogradsky Columns as an enrichment culture was an excellent source of mesophilic as well as thermophilic bacterial consortia that degrade cellulose and produce ethanol. A repeat using these environmental samples using the microaerophilic culture method could be done in the future to give a better comparison.

Thermophilic and mesophilic bacterial spore formers that degrade cellulose and produce ethanol under anaerobic conditions were isolated from mesophilic environments suggesting that thermophilic cellulose-degrading spore formers are ubiquitous and not restricted to thermophilic environments.

The instability of the bacterial consortia isolated may have been caused by the modified microaerophilic culture method used. The experiment was repeated using a few

samples with and without reducing agent to confirm the stability of the cellulose-degrading ability of the consortium after many sub-cultures.

The use of serum vials to screen environmental samples for efficient cellulose-degrading ability and ethanol production was beneficial. The heat treatment eliminated all non-spore formers while the anaerobic culture restricted growth to strict and facultative anaerobic bacteria that degrade cellulose/lignocellulose and uses the resulting hexose and pentose sugars to produce ethanol as an end-product. This preliminary selection was important with environmental samples that may not be extremely rich in the mixed bacteria of interest. Instead of consolidation the best cellulose-degrading consortia it may also be viable to consolidate the environmental samples for direct inoculation into the bioreactor. The selected consolidated consortium was used as inoculum for the mesophilic bioreactor, but the source inoculum for the thermophilic bioreactor was mixed herbivore dung collected and inoculated into the nutrient medium before being injected into the bioreactor. An advantage of using dung or even sewage is that the inoculum can be a much larger volume, which decreases the startup time required to increase the bacterial biomass load to the maximum capacity of granules. A problem with the microaerophilic culture method used was that the bacterial consortia selected in serum vials were not stable and did not retain efficient cellulolytic ability and the volume of the bioreactor inoculum was very small and not as high in bacterial density relative to the more direct route using dung.

It was, thus, not necessary to make a selection of anaerobic mixed bacterial consortia able to degrade lignocellulose efficiently and produce ethanol before bioreactor inoculation. Direct inoculation of a bioreactor with heat-treated cultured animal manure was found to work very well because these are a very rich source of inoculum.

The use of mixed bacterial consortia to degrade complex biopolymers such as lignocellulose has many benefits over the use of pure or defined cultures. In nature, lignocellulose is efficiently degraded by a community of mixed bacterial species with cooperative metabolic interactions that operate synergistically to produce ethanol. This process is much more efficient than using a single or co-culture and is better suited to a complex substrate such as lignocellulose, especially in a bioreactor system.

On an industrial scale, it would be very beneficial and cost-effective to use nutrient medium using tap water without autoclaving or a reducing agent but with indicator. The high bacterial load maintained in the bioreactor is the form of granules, ensured the exclusion of all competing microorganisms that entered the system. The presence of biofilm in the form of granules protected the strict anaerobes from being killed when aerobic nutrient medium entered the system. Facultative anaerobes were responsible for the aerobic nutrient medium immediately becoming anaerobic after entering the bioreactor.

Lignocellulose degradation for ethanol production can be achieved without the need for highly acid or basic hydrolysates that requires extensive cleanup. Bran has already been processed to produce the small particle size that forms a point of attachment for biofilm and granule formation. Other lignocellulosic waste would need to be milled into a pulp before used as a substrate for ethanol fermentation. The problems associated with using a solid substrate in a bioreactor system can be overcome by utilizing batch instead of continuous substrate feed and the use of multiple solids overflow filters to retain bran and granules.

In the semi-continuous AFBR nutrient medium was continuously supplied, which ensured liquid nutrients do not become a limiting factor as would be the case in batch fed fermentation. Enough bran substrate was fed in batch daily to fill the reactor vessel.

The amount differed depending on the rate of bran degradation, i.e. rate of reduction in bran bed height. A continuous feed of the bran substrate was not achieved because the solid substrate was prone to blocking the pipes and causing pipe bursts and constant interruption. Continuous nutrient and substrate feed are ideal for an AFBR. For this to be attained, the optimal HTR, nutrient feed rate and recycle rate and COD need to be determined. A lower HRT increases the rate of lignocellulose degradation (Figure 3.16) and subsequently, ethanol and hydrogen production.

The upward flow of the nutrient medium in the AFBR is ideal for keeping bacterial granules in suspension. The flow of nutrient medium allows the granules of bacteria attached to the bran flakes to have access to nutrients freely and continuously. The layer glass beads and activated charcoal helped to stimulate granule formation around the surface of gran particles.

The addition of the gas disengager was a key factor for maintaining a stable AFBR. The H2 gas production during the fermentation process needed to be removed from the recycle stream to prevent pressure from building in the system. Hydrogen and ethanol production were confirmed but further optimizing of bran feed rate, HRT, recycle rate and temperature.

Thermophilic cultures degraded lignocellulosic substrate faster than mesophilic cultures and produced higher levels of ethanol. In serum vial experiments and bioreactor experiments. A continuous thermophilic AFBR could be set up for the continuous distillation of ethanol, which would prevent product inhibition, which is problematic at high temperatures but not at mesophilic temperatures.

Many factors need to be optimized when performing DGGE. The PCR protocol must be optimized before DGGE can be performed. The factors affecting DGGE, such as

acrylamide concentration, various high and low urea concentrations, temperature, voltage and run time, had to be optimized to get the best separation and clarity of bands. The optimal conditions were as follows: 6% Acryl, 20-60% Urea, 17 h run time at 60°C, 100V with a 10 µl sample size.

Only the darker bands that were clearly separated from other bands could be excised. If the bands to too faint or too close together then it was not possible to excise the band. rDNA extraction from polyacrylamide DGGE gel slices. Incubating with sterile distilled water, the Quiex Gel DNA extraction kit (Quiegen) and the Crush and Soak method failed to extract DNA from the gel slices. Only DNA clean and concentrate kit (Zymo Research) successfully extracted the rDNA from the gel slices. PCR confirmed confirmation of the successful extraction of the correct size 16S rDNA segments. Even after verification of the correct size of the extracted rDNA, no proper sequences were obtained. The reason that the samples did not produce viable sequences may have been because the samples contained more than one different 16S rDNA sequence present or because of the contamination of DNA from one slice to another during excision of bands, slices were cut with too much gel between bands, thereby picking up more than one Template. The DGGE results confirmed that our consortia were made up of mixed bacterial species. Instead of DGGE a more current method of microbial diversity characterisation would be Metagenomic profiling (Kachienga *et al.,* 2019)

APPENDIX

MEDIA RECIPES APPENDIX A

1. DRCM Formula

	g/L
Peptone	10.000
Meat Extract	8.000
Yeast Extract·	1.000
Starch	1.000
Glucose	1.000
L-Cystein[1]	0.500
Sodium Acetate	5.000
Sodium Bisulfite	0.500
Ferric-Ammonium Citrate	0.500
Resazurin[2]	0.002
$_{dd}H_20$ to the volume of	1000 ml
Final pH 7.0 (±0.2)	

[1] Reducing agent
[2] Indicator (Goes from purple to pink to clear as the medium becomes anaerobic)

Source: (S. Robles *et al.*, 2000)

2. Endo Medium

	(g/L)
NH_4HCO_3	3.49
$NaHCO_3$	6.72
K_2HCO_3	0.7
$CaCl_2 \cdot 6H_2O$	0.3
$MgCl_2 \cdot 6H_2O$	0.1
$MnSO_4 \cdot H_2O$	0.015
$FeSO_4 \cdot 7H_2O$	0.025
$CuSO_4 \cdot 5H_2O$	0.005
$CoCl_2 \cdot 6H_2O$	0.0000124

Modified from (Endo, Noike and Matsumoto, 1982)

3. Minimal Medium

	(g/L)
K_2HPO_4	1
KH_2PO_4	1
$MgSO_4 \cdot 7H_2O$	0.5
NaCl	5
NH_4Cl	1
Whatman no. 1 filter paper	80 X 70 mm
Trace Metal solution (v/v)	1%
Yeast extract	0.5
Resazurin	0.002
$_{dd}H_20$ to the volume of	1000 ml
Trace Metal Solution	**(g/L)**
$MnSO_4 \cdot 2H_2O$	4.5
$FeSO_4$	0.01
$CaCl_2 \cdot 2H_2O$	0.1
$ZnSO_4 \cdot 7H_2O$	0.1
$CuSO_4 \cdot 5H_2O$	0.01
$CoCl_2$	0.183
$NaMoO_4$	0.0599
H_3BO_3	0.01
$_{dd}H_20$ to the volume of	1000 ml
Reducing agents	**(12.5% m/v)**
Cystein · HCL	1 ml/l
Hydrogen sulfide	1 ml/l

4. ATCC 1190 *Clostridium thermocellum* medium

with filter paper substituted for the glucose

ATCC Medium 1190	Amount
KH_2PO_4	1.5 g/L
$Na_2HPO_4 \cdot 12H_2O$	4.2 g/L
$MgCl_2 \cdot 6H_2O$	0.5 g/L
Yeast Extract	2.0 g/L
Whatman #2 filter paper: 10 ml medium in Hungate tube	8x70 mm
20 ml medium in serum vial	30 mm
Vitamin Solution (see below)	0.54 ml/L
Wolfe's Modified Mineral Elixir	5.0 ml/L
Resazurin (0.1%)	1.0 ml/L
Reducing Solution	40.0 ml/L
$_{dd}H_20$ to the volume of	1000 ml

A note of caution: This medium contains sodium sulfide, and hydrogen sulfide production will occur, primarily upon prolonged boiling. Hydrogen sulfide is hazardous, and preparation of this medium was done in a chemical fume hood. The medium was brought to a boil while gassing with a mixture of N_2. The solution was kept boiling for several minutes until the colour changed from blue to reddish-pink. The reducing solution was then added during continuous gassing and boiling. The pink colour disappeared, indicating reduction. Anaerobic (Hungate) technique was used to dispense the medium into tubes/vials and flushed with N_2/H_2 gas mixture. The tubes/vials were stoppered with butyl rubber, sealed and autoclaved for 20 minutes.

Reducing Solution	Amount
0.2 N NaOH	200.0 ml
$Na_2S \cdot 9H_2O$	2.5 g
L-Cysteine · HCl	2.5 g

The sodium hydroxide solution was brought to a boil while bubbling with N2. The solution was allowed to cool before the addition of sodium sulfide and cysteine. The solution was then transferred to a serum vial while being flushed by N_2. The solution was stoppered with butyl rubber, and autoclaved for 20 minutes.

Vitamin Solution	Amount
Biotin	20.0 mg
p-Aminobenzoic acid	50.0 mg
Folic acid	20.0 mg
Pantothenic acid calcium salt	50.0 mg
Nicotinic acid	50.0 mg
Vitamin B12	1.0 mg
Thiamine . HCL	5.0 mg
Pyridoxine hydrochloride	100.0 mg
Thioctic acid	50.0 mg
Riboflavin	5.0 mg
$_{dd}H_20$ to the volume of	500 ml

The vitamins were dissolved in 500 ml of distilled water. For 100 ml of medium, 0.5 ml of the mineral elixir was used. The bottle of vitamin solution was covered in foil to exclude light then stored in a refrigerator.

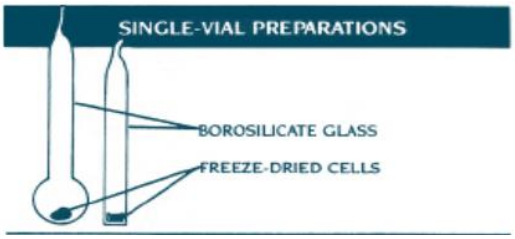
Wolfe's Modified Mineral Elixir	Amount
Nitrilotriacetic acid	1.5 g
$MgSO_4 \cdot 7H_2O$	3.0 g
$MnSO_4 \cdot H_2O$	500.0 mg
NaCl	1.0 g
$FeSO_4 \cdot 7H_2O$	100.0 mg
$Co(NO_3) \cdot 6H_2O$	100.0 mg
$CaCl_2$ (anhydrous)	100.0 mg
$ZnSO_4 \cdot 7H_2O$	100.0 mg
$CuSO_4$	10.0 mg
$AlK(SO_4)$ (anhydrous)	10.0 mg
Boric acid	10.0 mg
$Na_2MoO_4 \cdot 2H_20$	10.0 mg
Na_2SeO_3 (anhydrous)	1.0 mg
$_{dd}H_20$ to the volume of	1 L

The nitrilotriacetic acid was suspended in 500 ml water. It was dissolved by titrating with 2-3 N KOH until the pH is stabilized at 6.5. The rest of the ingredients were added and dissolved in the order they are listed. Finally, the volume was adjusted to 1000 ml. For 100 ml of medium, 0.5 ml of the mineral elixir was used. Source: www.atcc.org/Attachments/2455.pdf

ATCC

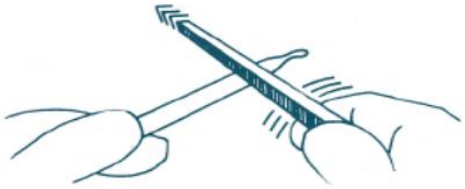
- All cultures should be regarded as potentially hazardous and should be opened by persons trained in microbiological techniques working in facilities with containment requirements appropriate for the biosafety level of the cultures.
- Work in a biological safety cabinet. If this is not possible, wear suitable eye protection. Hold vials away from face and over a can or tray
- Wear gloves
- Sterilize all empty vials and fragments before disposal.

REVIVING FREEZE DRIED CULTURES

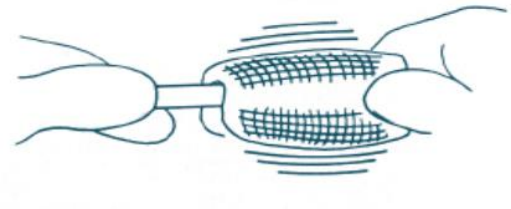


OPENING THE VIAL

1 These preparations may be enclosed in a thin skin of cellulose. This skin must be removed [either with a sharp blade or by soaking in water for a few minutes, score the ampule once briskly with a sharp file about one inch from the tip.



2 Disinfect the ampule with alcohol-dampened gauze.



3 Wrap gauze around the ampule and break at the scoring area. Care should be taken not to have the gauze too wet, or alcohol could be sucked into the culture when the vacuum is broken. Rehydrate material at once

Bacteria and Algae

Aseptically add 0.5 ml of the liquid medium to the freeze-dried material with a sterile Pasteur pipette and mix well. For bacteria transfer the total mixture to a test tube containing 5 to 6 ml of the recommended broth medium. The last few drops of this suspension also may be transferred to an agar slant. Algal cultures must be initiated on agar plates.

Incubate cultures under the appropriate conditions. Given proper treatment and conditions, most freeze-dried cultures will grow out in a few days. However, some may exhibit a prolonged lag period and should be given twice the normal incubation time before discarding a nonviable.

SOLUTIONS FOR MOLECULAR WORK APPENDIX C

1. 5X TBE buffer

10 ml 50X TBE (BIO-RAD)

90 ml $_{dd}H_2O$

2. 2X PCR Master Mix Composition

0.05units/µl Taq DNA Polymerase (recombinant) in reaction buffer

4mM $MgCl_2$

0.4mM of each dNTPs (dATP, dCTP, dGTP, dTTP)

3. Running buffer for Agarose gel electrophoresis (0.5X TBE)

100 ml 5X TBE

10 µl ethidium bromide

$_{dd}H_2O$ to the volume of 1000 ml

4. O'GeneRuler™ 100 bp DNA Lader Plus, ready to use

Composed of fourteen chromatography-purified individual DNA fragments (in base pairs); 3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 in:

10mM Tris-HCl (ph 7.6)

0.15% orange G

0.03% xylene cyanol FF

60% glycerol

60mM EDTA

5. 6 X DNA Loading Dye (Supplied with O'GeneRuler ladders)

10mM Tris-HCl (ph 7.6)

10 mM EDTA

0.025% orange G

0.005% xylene cyanol FF

10% glycerol

6. 1 and 1.7% Agarose gels

1% Agarose Gel	1.7% Agarose Gel
0.5g Agarose	1.7g Agarose
10ml 5X TBE buffer	20ml 5X TBE buffer
40ml $_{dd}H_20$	80ml $_{dd}H_20$
Heat until agarose is completely dissolved, then add 1µl ethidium bromide	

DGGE Solutions

Only Bio-Rad chemicals and solutions were used.

7. 100% and 0% Denaturing Solutions

	100% Denaturing Solution		0% Denaturing Solution	
	6 % Gel	8 % Gel	6 % Gel	8 % Gel
40% Acrylamide/Bis solution (Bio-Rad)	15 ml	20 ml	15 ml	20 ml
50X TAE buffer (Bio-Rad)	2 ml	2 ml	2 ml	2 ml
Formamide	40 ml	40 ml	-	-
Urea	42 g	42 g	-	-
$_{dd}H_20$	-	-	83 ml	78 ml
Total volume	to 100 ml	to 100 ml	100 ml	100 ml

The solution was covered in foil to exclude light and stored at 4°C

8. Ammonium Persulfate

0.1 g Ammonium Persulfate

$_{dd}H_20$ to 1ml

(Stored at -20°C for about a week)

9. 1X TAE Running Buffer

140 ml 50X TAE buffer

6 860 ml $_{dd}H_20$ (final volume 7 000ml)

10. Protocol: Extraction of DNA fragments from Polyacrylamide Gels

Important points before starting:

- The yellow color of Buffer QX1 indicates a pH ≤7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- A heating block or water bath at 50°C is required.
- 3 M sodium acetate, pH 5.0, may be necessary.
- All centrifugation steps are at maximum speed (≥10,000 x *g*, ~13,000 rpm) in a conventional, table-top microcentrifuge.
- Prepare diffusion buffer: 0.5 M ammonium acetate; 10 mM magnesium acetate; 1 mM EDTA, pH 8.0; 0.1% SDS.
- A disposable plastic column or a syringe barrel containing either a Whatman® GF/C filter or packed, siliconized glass wool will be required for each extraction.

Procedure

1. **Excise the gel slice containing the DNA band with a clean, sharp scalpel.**
   Minimize the size of the gel slice by removing excess polyacrylamide.
2. **Weigh the gel slice. Add 1–2 volumes of diffusion buffer to 1 volume of gel (i.e., 100–200 µl for each 100 mg of gel).**
3. **Incubate at 50°C for 30 min.**
4. **Microcentrifuge the sample for 1 min.**
5. **Carefully remove the supernatant using a pipet or a drawn-out Pasteur pipet. Pass the supernatant through a disposable plastic column or a syringe containing either a Whatman GF/C filter or packed, siliconized glass wool to remove any residual polyacrylamide.**
6. **Calculate the approximate volume of the recovered supernatant.**
7. **For DNA fragments <100 bp, add 6 volumes of Buffer QX1 to 1 volume of sample. Otherwise, add 3 volumes Buffer QX1. Check that the color of the mixture is yellow.**
   If the color of the mixture is orange or purple, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color should turn to yellow.
   The adsorption of DNA to QIAEX II particles is only efficient at pH ≤7.5. Buffer QX1 now contains a pH indicator which is yellow at pH ≤7.5, and orange or violet at higher pH, allowing easy determination of optimal pH for DNA binding.
8. **Resuspend QIAEX II by vortexing for 30 s.**

9. **Add 10 µl of QIAEX II and mix. Incubate at room temperature (15–25°C) for 10 min. Vortex every 2 min to keep QIAEX II in suspension.**
10. **Centrifuge the sample for 30 s and remove supernatant.**
11. **Wash the pellet twice with 500 µl of Buffer PE.**
12. **Air-dry the pellet for 10–15 min or until the pellet becomes white.**

    Do not vacuum dry, as this may cause overdrying. Overdrying the QIAEX II pellet may result in decreased elution efficiency.
13. **To elute DNA, add 20 µl of 10 mM Tris·Cl, pH 8.5, or H_2O and resuspend the pellet by vortexing. Incubate for 5 min at room temperature.**

    Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water for elution, make sure that the pH is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
14. **Centrifuge for 30 s. Carefully transfer the supernatant into a clean tube.**

    The supernatant now contains purified DNA.
15. **Optional: repeat steps 13 and 14 and combine the eluates.**

    A second elution step will increase the yield by approximately 10–15%.

11. Crush and Soak Protocol

(DNA Fragment Purification from Acrylamide)

Use a hot needle to make a hole in the bottom of a 0.5ml microcentrifuge tube. The gel slice is placed into a microfuge tube and in turn into a 1.5ml tube. The assembly is centrifuged at top speed (14k rpm) for 1-5 minutes, or until the entire gel slice is collected in the lower tube.

Crush and Soak

14. A sufficient amount of buffer is added to the gel pieces to cover them.
15. This slurry is incubated at 37° C with shaking for 2-12 hours. Longer incubations give better recoveries and are essential for the acceptable recovery of fragments larger than 500bp.
16. Spin in the microfuge for 10 minutes at 14,000 rpm.
17. Remove the eluate to a 1.5 ml microcentrifuge tube
18. Add another 500 µl of crush and soak solution.
19. Repeat the spin and pool the recovered supernatant.
20. Polyacrylamide fragments carried over into the supernatant may be recovered by a second centrifugation step, or by filtration through a 0.2mm filter.
21. Add 10 µg of Glycogen and 2.5 volumes of 100% Ethanol.
22. Chill the samples at -20°C for approximately 30 min.
23. Centrifuge for 5 min at maximum speed in a microcentrifuge to pellet the DNA.
24. Discard the supernatant and resuspend the DNA pellet in 200 µl of TE Buffer.
25. Add 25 l of 3 M Sodium Acetate and 750 µl of 100% Ethanol. Repeat Steps #9 and #10.
26. Discard the supernatant and resuspend the DNA pellet in 80 µl TE Buffer, using two 40 µl aliquots.
27. Store the DNA at -20°C.

Solutions

Crush and Soak Solution (Use diffusion buffer from Quiex kit)

NH_4Ac (10.0M) 5.0ml 0.5M
MgAc (1.0M) 1.0ml 10mM
EDTA (0.5M) 0.2ml 1mM
10% SDS 1.0ml 0.1%
ddH_2O 92.8ml

Sterile filter (NH_4Ac is volatilized by autoclaving)

3 M NaOAc pH 5.2

24.6 g anhydrous sodium acetate

pH to 5.2 with acetic acid and bring up to 100 ml with ddH_2O

store at room temperature

TE Buffer

10 mM Tris-HCl, pH 8.0
1 mM EDTA

Other Reagents

DMCS treated glass wool (Alltech Assoc. Inc. #4037, 50 g)

0.22 µm disposable micro tip filters (syringe type)

12.

DNA Clean & Concentrator™-100

Catalog Nos. **D4029 & D4030**

PROTOCOL

Add 2-7 volumes of **DNA Binding Buffer** to each volume of DNA sample (see table below). Mix briefly by gently inverting the tube.

Application	DNA Binding Buffer : Sample	Example
Plasmid, genomic DNA (>2 kb)	2 : 1	200 µl : 100 µl
PCR product, DNA fragment	5 : 1	500 µl : 100 µl
ssDNA (*e.g.* cDNA, M13 phage[1])	7 : 1	700 µl : 100 µl

Use any of the following three procedures to process samples.

Microcentrifuge Protocol (for sample/**DNA Binding Buffer** mixtures ≤ 600 µl)

1. Remove **Reservoir** from **Zymo-Spin™ V Column** and transfer the sample mixture to the **Zymo-Spin™ V Column** placed inside a **Collection Tube.**
2. Centrifuge at maximum speed (≥10,000 x *g*) for 1 minute. Discard the flow-through.
3. Add 600 µl **DNA Wash Buffer** to the **Zymo-Spin™ V Column**. Centrifuge at maximum speed for 1 minute. Discard the flow-through and repeat wash step.
4. Discard flow through. Place **Zymo-Spin™ V Column** into a **Collection Tube** and centrifuge at maximum speed for 30 seconds to remove any residual wash buffer.
5. Transfer the **Zymo-Spin™ V Column** into a new 1.5 ml microcentrifuge tube. Add 150 µl **DNA Elution Buffer**[3] or water[4] directly to the column matrix in the **Zymo-Spin™ V Column**. Wait for one minute to ensure that the column matrix has been fully hydrated prior to centrifugation at maximum speed for 1 minute to elute DNA.

   Ultra-pure, concentrated DNA is now ready for use.

Centrifuge Protocol

1. Transfer the sample mixture to the **Zymo-Spin ™ V Column/Reservoir assembly**[1] inside a 50 ml conical tube.

   Note: *Ensure the connection between the* ***Zymo-Spin ™ V Column*** *and* ***Reservoir*** *is finger-tight prior to use.*
2. Centrifuge at 500 x *g* for 5 minutes. Discard flow-through.
3. Add 2 ml **DNA Wash Buffer** to the **Reservoir**. Centrifuge at 500 x *g* for 5 minutes. Discard flow-through. Repeat wash step.
4. Transfer the **Zymo-Spin™ V Column** to a **Collection Tube** and, using a microcentrifuge, centrifuge at maximum speed (≥10,000 x *g*) for 30 seconds to remove any residual wash buffer.
5. Transfer the **Zymo-Spin™ V Column** into a new 1.5 ml microcentrifuge tube. Add 150 µl **DNA Elution Buffer**[2] or water[3] directly to the column matrix in the **Zymo-Spin™ V Column**. Wait for one minute to ensure that the column matrix has been fully hydrated prior to centrifugation at maximum speed for 1 minute to elute DNA.

   Ultra-pure, concentrated DNA is now ready for use.

Reagaents, Equipment and Suppliers APPENDIX D

List of chemicals and Suppliers

Supplier	Reagents
Merck	Yeast extract powder, Na_2HPO_4, $MnCl_2{\cdot}H_20$, $MgCl_2{\cdot}6H_{20}$, K_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$,NaCl, NH_4Cl, Resazurin tablets, $MnSO_4 \cdot 2H_2O$, $FeSO_4$, $CaCl_2 \cdot 2H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $CoCl_2$, $NaMoO_4$, H_3BO_3, $C_3H_7NO_2S$ HCL H_2O, H_2S, NH_4HCO_3, $NaHCO_3$, K_2HCO_3, $CaCl_2 \cdot 6H_2O$, $MgCl_2 \cdot 6H_2O$, $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $Na_2HPO_4 \cdot 12H_2O$, $FeSO_4 \cdot 7H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $CaCl_2$ (anhydrous), $CuSO_4$, $AlK(SO_4)$ (anhydrous), $Na_2MoO_4 \cdot 2H_20$, Na_2SeO_3 (anhydrous), Ethanol (absolute), Glycerol,
Sigma Aldrich	Resazurin indicator certified, Biotin, p-Aminobenzoic acid, Folic acid, Pantothenic acid calcium salt, Nicotinic acid, Vitamin B12, Thiamine. HCL, Pyridoxine hydrochloride, Thioctic acid, Riboflavin, Nitrilotriacetic acid, 120 ml serum vials with butyl rubber stoppers and aluminium lids, Wheaton crimper/ decrimper
BIO-RAD	Ethidium bromide (10g/ml), 50X TBE, 40% Acrylamide/Bis (37.5:1), 50X TEA buffer, Formamide, Urea crystals (100% deionised), Ammonium persulfate, TEMED,
Whitehead scientific	Agarose
Fermentas	2X PCR Master Mix**,** O'GeneRuler™ 100 bp DNA Lader Plus, ready to use, 6X DNA Loading Dye (Supplied with O'GeneRuler ladders)
Fluka	Resorcinol
Inqababiotech	Fungal/Bacterial DNA extraction kit, Soil DNA extraction kit, DNA clean and concentrate kit,

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