COMPARATIVE ANALYSIS OF BIOHYDROGEN PRODUCING BACTERIAL CONSORTIA IN THREE THERMOPHILIC ANAEROBIC FLUIDISED BED BIOREACTORS

Keneilwe Mmule Sebola

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, in partial fulfilment of the requirements for the degree of Master of Science in Medicine (Pharmaceutical Affairs).

Johannesburg 2012

DECLARATION

I, Keneilwe Mmule Sebola, declare that this research report is my own work. It is being submitted in partial fulfilment of the requirements for the degree of Master of Science in Medicine (Pharmaceutical Affairs) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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DEDICATION

This research report is dedicated to my Lord, Jesus Christ for granting me the strength and affording me the opportunity to do this research project. To my family for their compromise, support and their encouragement during the duration of my studies

ABSTRACT

Global warming has stimulated research into alternative energy carries and fuels. Hydrogen is one of these alternative fuels that are recognized as a promising future energy source. Historically, it is produced by water electrolysis and the gasification of coal. Hydrogen is a natural though transient by-product of several microbial driven biochemical reactions, including anaerobic digestion and fermentation. Microorganisms degrade complex molecules to produce butyrate and alcohols with CO_2 and hydrogen as the only by- products. Hydrogen produced by microorganisms is known as biohydrogen.

This study aimed to identify biohydrogen- producing bacteria in three Anaerobic Fluidised Bed Bioreactors (AFBRs), which are capable of producing hydrogen under anaerobic and thermophilic conditions, by using PCR-DGGE analysis of the 16 S rDNA genes. Sewage sludge from anaerobic digester and cow- dung were used as inoculum to isolate potential H_2-producing organisms. The operational conditions were manipulated to removing mesophilic bacteria and non- spore forming bacteria by gradually increasing the temperature to thermophilic conditions and the pH maintained at acid conditions to allow acidotolerant bacteria to survive. The bioreactors were operated for a period of forty days for each research cycle. Bioreactor One was operated at 55°C for eight days and then the temperature kept at a constant 65°C for the remainder of the research cycle. Bioreactor Two was operated for 8 days at each temperature ranging from 45°C - 65°C, with increments of 5 °C. Bioreactor Three was operated at each temperature for ten days ranging from 55- 70°C, also with increments of 5°C. Samples for microbial community identification were taken at 55°C and 65°C. The bacterial morphologies and structural properties were evaluated by examining the hydrogen- producing granules, isolated at 65°C, using scanning electron microscopy. Species of the families *Bacillus*, *Enterobacteria*, *Actinomyces*, *Clostridium* and *Veillonella* were identified. *Clostridium thermopalmarium*, *Bacillus coagulans* and *Bacillus thermoamylovorans* were the culturable species at the desired operational temperature of 65°C. *C. thermopalmarium* and *B. coagulans* are major H_2 producers with theoretical values of 4mol H_2/ g glucose and 3mol H_2/ mol sugar, respectively. Sewage sludge is the best source of biohydrogen producing bacteria in comparison to cow dung as an inoculum in AFBRs. Bioreactor 1 with a constant operational temperature of 65°C and at a HRT of 6.5 allowed for the desired *Clostridium* sp. to be the predominant H_2 producer.

ACKNOWLEDGEMENTS

I would like to extend my earnest gratitude towards the following:
Prof Vincent Gray (Molecular and Cell Biology, University of the Witwatersrand) for his support, expertise, guidance and granting me the opportunity to undertake this project.

Prof Sandy Van Vuuren, (Pharmacy and Pharmacology, University of the Witwatersrand) for her support, guidance and constant encouragement.

The Olifantsvlei Waste Water Treatment Works and Regional sludge handling facility (Soweto) and the Animal and Dairy Science Research Institute (Irene, Pretoria) for the inocula.

Inqaba Biotechnical Laboratories, South Africa, and the University Stellenbosch DNA sequencing unit, South Africa, for their efficient DNA sequencing process.

A special thank you to Mr Abe Seema of the Electron Microscopy Unit (University of the Witwatersrand) for training and assistance in the use of the Scanning Electron microscope.

Lastly I would like to thank my colleague Phumlani Masilela of the Hyvolution research group under the supervision of Prof Vincent Gray, for his assistance with the PCR-DGGE procedure in the initial stages of the project.

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- 16SrDNA: 16 S ribosomal DNA
- AFBR: Anaerobic Fluidized Bed Bioreactor
- $CaCl_2$: Calcium Chloride anhydrous
- $CaCl_2 2H_2O$: Calcium Chloride dihydrate
- CO: Carbon monoxide
- CO_2: Carbon dioxide
- $CoCl_2$ $6H_2O$: Cobalt Chloride
- $CuSO_4$ $5H_2O$:Copper Sulphate
- DGGE: Denaturing Gradient Gel Electrophoresis
- dNTPs: Deoxyribonucleotides Triphosphates
- Fe SO_4 $7H_2O$: Ferrous Sulphate
- H_2: Hydrogen
- H_2O: Water
- HPR: Hydrogen Producing Rate
- HRT: Hydraulic Retention Rate
- K_2HPO_4: Dipotassium orthophosphate
- KH_2PO_4: Potassium dihydrogen phosphate
- $MgCl_2$ $6H_2O$: Magnesium Chloride
- $MgSO_4.7H_2O$: Magnesium Sulphate hepahydrate
- $MnSO_4H_2O$: Manganese Sulphate
- $NaHCO_3$: Sodium Hydrogen Carbonate
- $Na_2HPO_4.2H_2O$: Di sodium Orthophosphate dihydrate
- NH_4Cl: Ammonium Chloride
- NH_4CO_3: Ammonium Carbonate
- PCR: Polymerase Chain Reaction
- PNS: Purple Non-sulphur bacteria
- SEM : Scanning Electron Microscopy
- TAE: Tris Acetate Ethylenediaminetetraacetic acid

CHAPTER 1: INTRODUCTION

1.1 **Hydrogen as alternative energy carrier**

With global warming becoming a growing concern and soaring concerns on environmental deterioration (Zhang(b) *et al*., 2008), countries are under pressure to find alternative energy sources that are sustainable and efficient. This state of affairs has stimulated research into alternative energy carries and fuels (Maintinguer *et al.,* 2008). Hydrogen is one of the alternative fuels that is recognized as a promising future energy source (Koskinen *et al*., 2007; Wu *et al*., 2008; Zhang(a) *et al*., 2008; Zhang(b) *et al*., 2008; Valdez-Vazquez and Poggi-Varaldo, 2009). Hydrogen is an ideal clean energy source for the future because of its high conversion efficiency, recyclability and non- polluting nature (Fang(a) *et al*., 2002 ; Fang(b) *et al*., 2002;Lin *et al*., 2006; Maintinguer *et al.,* 2008; Valdez-Vazquez and Poggi-Varaldo*,* 2009). It is also called a clean fuel because it does not produce carbon dioxide, sulphur oxides or nitrogen oxides that cause air pollution during combustion (Valdez-Vazquez and Poggi-Varaldo*,* 2009). It can be used in either fuel cells or fuel engines to generate power without producing greenhouse gas emission (Chang *et al*, 2008), releasing only water when combusted (Zhang *et al*., 2003). Many experts have proposed that the hydrogen economy be introduced, but before the hydrogen economy becomes a reality, the sustainable production of hydrogen should be extensively developed (Valdez-Vazquez and Poggi-Varaldo*,* 2009).

1.2 **Commercial importance of hydrogen**

Hydrogen is one of the oldest known molecules in the Universe and is used extensively by many industries for a variety of applications (Ramachadran and Menon, 1998). Most of its use is based on its reactivity rather than its physical properties (Ramachadran and Menon, 1998). It is an important industrial commodity (Zhang *et al*., 2003). It is widely used for the synthesis of ammonia, alcohols and aldehydes as well as the hydrogenation of edible oils, petroleum, coal and shale oil (Fang(b) *et al*., 2002; Zhang *et al*., 2003). Recently, its use in petroleum refining has been growing rapidly due to a combination of factors relating to changes in crude, environmental regulations such as limits of sulphur in diesel, allowable limits to nitrogen and sulphur compounds in off- gas emissions to the atmosphere, aromatic and light hydrocarbon concentration in gasoline etc (Ramachadran and Menon, 1998).

1.3 **Conventional methods for hydrogen production**

Hydrogen can be generated from H_2O, biomass, natural gas or (after gasification) coal (Turner, 2004).Today approximately 95% of commercial hydrogen is generated by steam reforming natural gas and gasification of coal (Turner, 2004; O-Thong *et al*, 2009) and from fossil fuels such as naphtha (Valdez-Vazquez and Poggi-Varaldo, 2009). However, this practice is an environmentally self-defeating contradiction since a clean fuel is generated from fossil fuels resulting in pollution and consumption of limited energy sources (Valdez-Vazquez and Poggi-Varaldo, 2009). It is also traditionally produced by hydrocarbon reformation or electrolysis of water, but it is technically feasible to harvest hydrogen produced by microorganisms (Fang(b) *et al*., 2002; Zhang *et al*., 2003).

1.4 **Hydrogen production by microorganisms**

In terms of global environmental considerations, microbial fermentation of hydrogen from renewable organic waste sources represents an important aspect for both energy generation and waste management (Chang *et al*., 2008). Hydrogen is a natural though transient by-product of several microbial driven biochemical reactions, including anaerobic digestion and fermentation (Fang(b) *et al*., 2002; Chang *et al*., 2008). The production of hydrogen through biological processes is environmentally friendly, cost effective and less energy intensive when compared to the hydrogen production from thermochemical and electrochemical processes (Lin and Chang, 1999; Levine et al., 2004; Lin *et al*., 2006; Hung *et al*., 2007; Wu *et al*., 2008; Zhang (a) *et al*., 2008). Hydrogen production by microorganisms can be divided into two major categories, the first being hydrogen production by light- requiring anaerobic photosynthetic organisms and anaerobic bacteria that produce hydrogen by fermentation metabolism (Ueno *et al*., 2001). Hydrogen production by microorganisms through fermentation metabolism is known as dark fermentation. Dark fermentation has a higher hydrogen production rate than other hydrogen production methods such as photo fermentation (Lay *et al*., 2010). Dark fermentation requires a carbohydrate- rich substrate, thus wastewaters and other biomass serve as good potential nutrient sources (Ueno *et al*., 2001; Levine et al., 2004). Biological hydrogen production by dark fermentation of organic wastes can reduce waste disposal problems and decrease substrate costs (Ueno *et al*., 2001; Lin *et al*., 2006; Koskinen *et al*., 2007; Chang *et al*., 2008; Zhang *et al*., 2008) as these wastewaters or organic waste solids are renewable and cheap sources which are abundant (Levine et al., 2004; Valdez-Vazquez and Poggi-Varaldo, 2009). This is considered the most commercially feasible biological means of producing hydrogen, due to the high hydrogen production rate (HPR) with the additional benefit of waste reduction (Wu *et al*., 2008; O-Thong *et al*., 2009). Conventional wastewater treatment technologies employ the methane production ability of anaerobes that convert organic pollutants in wastewater into methane through a two step process (Fang(a) *et al*., 2002). In the process pollutants are hydrolysed and acidified in the first reactor, thus favouring acidoogenic bacteria, which produce hydrogen as a by product (Fang(a) *et al*., 2002). The acidic effluent is then treated in a second methanogenic reactor, with methane being the by product of this second step (Fang(a) *et al*., 2002).

1.4.1 **Dark fermentation**

Dark fermentation also known as anaerobic digestion, is considered to have two separate and distinct phases brought about by different populations of bacteria: a) the non methanogenic phase, where a variety of anaerobic bacteria transform complex substrates into a variety of soluble and gaseous fermentation products including acetate, propionate, butyrate, carbon dioxide and hydrogen; and b) the methanogenic phase, where methanogenic bacteria utilize acetate, carbon dioxide and hydrogen as substrates in methanogenesis (Grant and Long, 1981). Under methanogenic conditions characterized by low hydrogen partial pressures various syntrophic bacteria are able to oxidize acetate, propionate and butyrate into carbon dioxide and hydrogen. In most anaerobic environments, the hydrogen consumption is carried out very quickly by different microbial groups (Valdez-Vazquez and Poggi-Varaldo, 2009). Contrary to this natural fact, our interest is to proliferate the hydrogen accumulation in order to use it as a fuel (Valdez-Vazquez and Poggi-Varaldo, 2009). Therefore, hydrogen accumulation is linked with the inhibition of hydrogen- consuming microorganisms (Valdez-Vazquez and Poggi-Varaldo, 2009). In addition, it has been demonstrated that thermophilic dark fermentation hydrogen production gives a higher reaction rate and better process performance as it results in increased energy recovery and decreased problems with contaminating hydrogen- consuming microorganisms (O-Thong *et al*., 2009).

1.4.2 **Microorganisms capable of producing hydrogen**

Among a large number of microbial species, strict anaerobes and facultative chemoheterotrophs such as clostridia and enteric bacteria are efficient hydrogen producers (Hung *et al*, 2007). The members of the genus *Clostridium* are known for evolving hydrogen during anaerobic fermentation (Fang(b) *et al*., 2002; Chang *et al*., 2008).

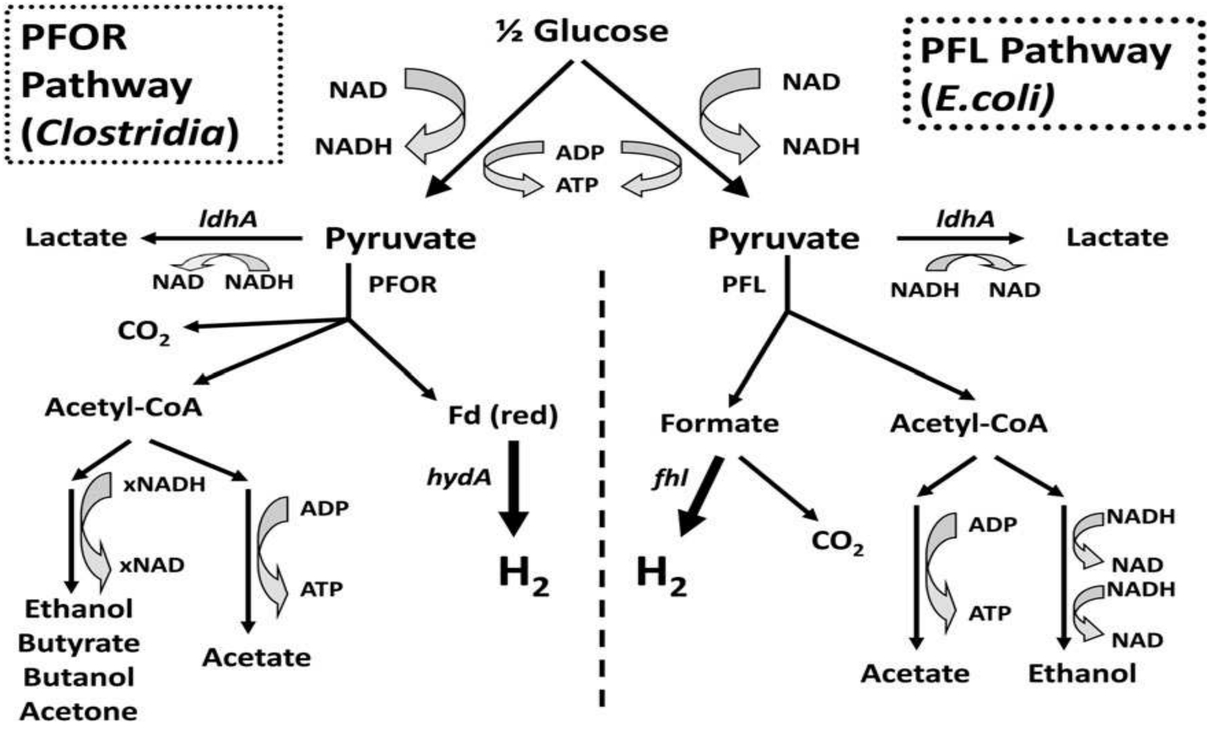


Figure 1: Metabolic Pathways in H_2 Production (Hallenbeck and Ghosh, 2010)

Two major H_2 production pathways exist. One carried out typically by facultative anaerobes and the other by obligate anaerobes (Hallenbeck and Ghosh, 2010). In both cases glucose is broken down to pyruvate through the glycolytic pathway. However, pyruvate degradation differs with the PFL (pyruvate: formate lyase) pathway carried out typical of facultative anaerobes, such as *Escherichia coli*, shown on the right part of Figure1, and the PFOR (pyruvate: ferredoxin oxidoreductase) pathway, typical for strict anaerobes, such as Clostridia, shown on the left part of Figure1 (Hallenbeck and Ghosh, 2010). Many organisms contain both systems, however, only one plays a predominant role in fermentation. In the PFOR system, additional hydrogen can be derived from electrons from NADH through several different mechanisms (Hallenbeck and Ghosh, 2010). In the PFL system, there is no mechanism for producing hydrogen from the generated NADH. In both systems, ATP is produced when acetate is made from acetyl-CoA, and both types must have means to oxidize NADH to regenerate the NAD necessary for continuing glycolysis (Hallenbeck and Ghosh, 2010). In the PFL system, this is usually through production of ethanol. In the PFOR system (Clostridia), a variety of reduced products; ethanol, butanol, butyrate, acetone, are possible, but the individual pathways are not shown in Figure 1.ldhA, lactate dehydrogenase, if present and active, can drain away pyruvate that otherwise could be used for producing hydrogen. As well, minor pathways, e.g. succinate production from phosphoenolpyruvate (frdBC) in *E. coli*, are not shown (Hallenbeck and Ghosh, 2010). It has been shown that the [Fe] -

hydrogenase of *Clostridium* spp. could dispose of excess electrons to produce hydrogen gas during fermentative metabolism (Chang *et al*., 2008). Sung *et al.* (2002), used terminal restriction fragment length polymorphism (T-RFLP) to identify hydrogen- producers mixed communities in mesophyllic continuous flow reactors using sucrose and heat-treated inocula (Sung *et al.* 2002; Valdez-Vazquez and Poggi-Varaldo, 2009). The results indicated that two major groups of *Clostridium* species were dominant during the first 15 days of operation (Valdez-Vazquez and Poggi-Varaldo, 2009). The first group being composed of *C. beijerinckii*, *C. botulinum*, *C. putrificum* and *C. sporogenes*. The second dominant species was identified as *C. butyricum* (Valdez-Vazquez and Poggi-Varaldo, 2009). The authors found that a decrease in hydrogen production was accompanied with a decrease in the total *Clostridium* species and vice versa. Since the most commonly formed metabolites during hydrogen fermentation are acetate, propionate, butyrate, ethanol and butanol, several authors have suggested that *Clostridium* species are predominantly present in hydrogen producer systems (Lin and Chang, 1999; Nath and Das, 2004; Valdez-Vazquez and Poggi-Varaldo, 2009; Hallenbeck and Ghosh, 2010). Fang(a) *et al* (2002) found that 69.1% of the clones isolated from a granular sludge hydrogen producing reactor operated at room temperature were Clostridium spp., whereas Fang(b) *et al*., (2002) found that only 64.6% of the clones isolated from mesophilic hydrogen producing sludge were Clostridium spp. With Enterobacteriaceae only accounting for 18.8% of the clones retrieved from the sludge (Fang(a) *et al*., 2002; (Fang(b) *et al*., 2002).

1.4.3. **Microbial ecology of bacteria involved in organic matter degradation in nature**

Bacterial communities in nature play a key role in the production and degradation of organic matter, cycling of nitrogen, sulphur and many metals. Bacterial communities are also involved the degradation of many environmental pollutants (Davey and O'Toole, 2000). Most natural degradation processes require the concerted effort of bacteria with different metabolic capabilities, and it is likely that bacteria reside within communities carry out many of the complex processes (Schink, 1997; Davey and O'Toole, 2000). These multispecies microconsortia can result from an association between metabolically cooperative organisms, and their proximity facilitates interspecies substrate exchange and the removal or distribution of metabolic products (Schink, 1997; Davey and O'Toole, 2000). For example, the degradation of the complex organic matter into methane or carbon dioxide during anaerobic digestion requires the interaction of at least three guilds (Bryant, 1979; Smith *et al*., 1979;

Schink, 1997; Davey and O'Toole, 2000). Guilds are metabolically related bacterial community, for example all fermentative bacteria form one guild.

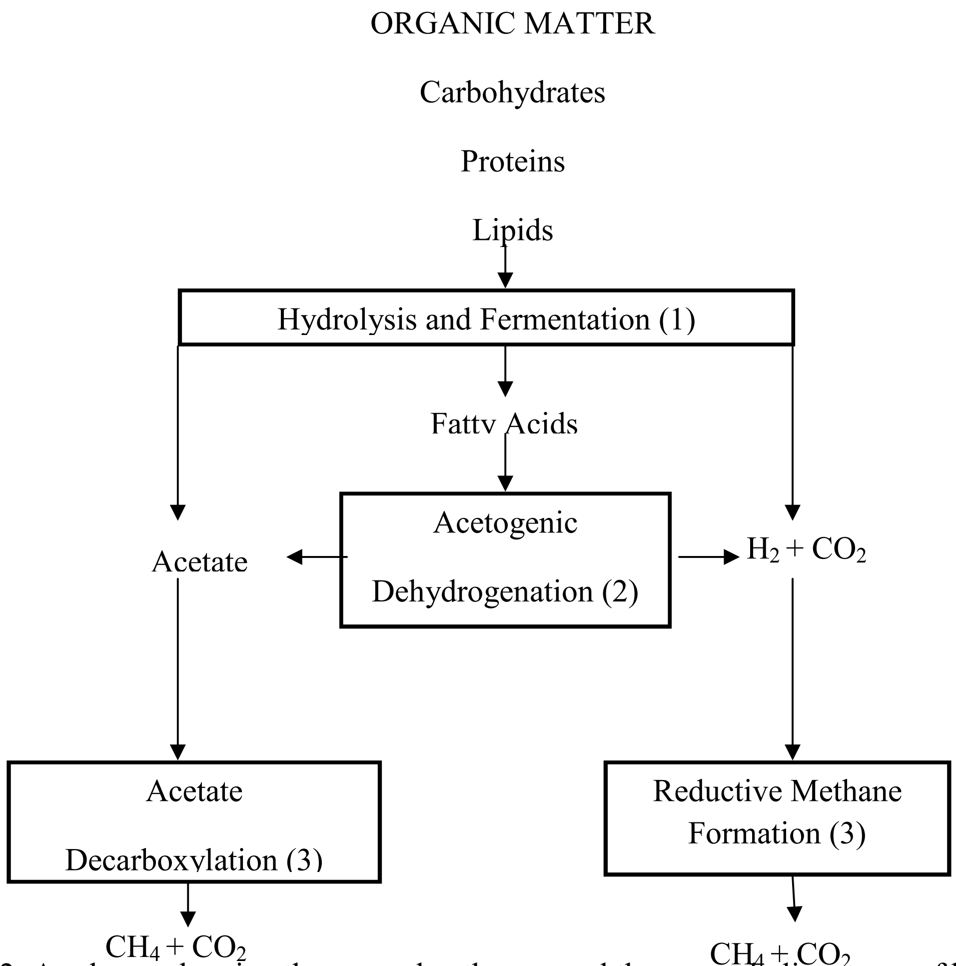


Figure 2: A scheme showing the general pathways and three metabolic groups of bacteria involved in complete anaerobic degradation of organic matter (Adapted from Bryant, 1979; Schink, 1997).

Fermentative bacteria initiate the catabolism, producing acids and alcohols that are then utilised as substrates by acetogenic bacteria (Lin and Chang, 1999; Davey and O'Toole, 2000). Finally, methanogens obtain energy from converting acetate, carbon dioxide and hydrogen to methane. Methane fermentation occurs in environments that O_2, sulphates and nitrates are not easily accessible (Bryant, 1979). It usually involves two types of microbes, dictated by temperature, thermophilic species active at 45° C to 70°C and mesophilic species present at lower temperature (Bryant, 1979). The fermentation proceeds at a pH range of 5 to 8 (Bryant, 1979).

While the three fermentation stages can be separated in the scheme in reality the efficiency of metabolism of the three guilds of microorganisms (1, fermentative bacteria; 2, obligate H_2-forming, acetogenic bacteria; 3, methanogenic bacteria, Figure 2), are highly dependent on each other, as the later members of the food chain depend on the earlier members for their substrate (Bryant, 1979; Smith *et al*., 1979; Schink, 1997). The late members of the food chain exert influence on the earlier members by removing metabolic products, thus creating a mutual dependency (Schink, 1997). Schink (1997) stated that there are in actual fact four guilds of bacteria involved in the degradation of organic matter, primary fermenters, secondary fermenters and two types of methanogens (Schink, 1997). Primary fermenters convert polymers into oligomers and monomers through extracellular hydrolytic enzyme action, the resulting mononers are further degraded into and fatty acids, succinate, lactate and alcohols. Some of these fermentation products, especially acetate, H_2 and CO_2 are converted directly into methane by methanogens. Secondary fomenters degrade the resultant fatty acids, succinate, lactate and alcohols into actetate, H_2, CO_2 and often formate (Schink, 1997). These are then used by methanogens.

Due to the very efficient cooperations and mutual dependence required for methane fermentation the bacterial members involved can form a biofilm. In fact, biofilms provide and ideal environment for the establishment of syntrophic relationships. Syntrophism is a special case of symbiosis in which two metabolically distinct types of bacteria depend on each other to utilize certain substrates, typically for energy production (Schink, 1997; Davey and O'Toole, 2000).

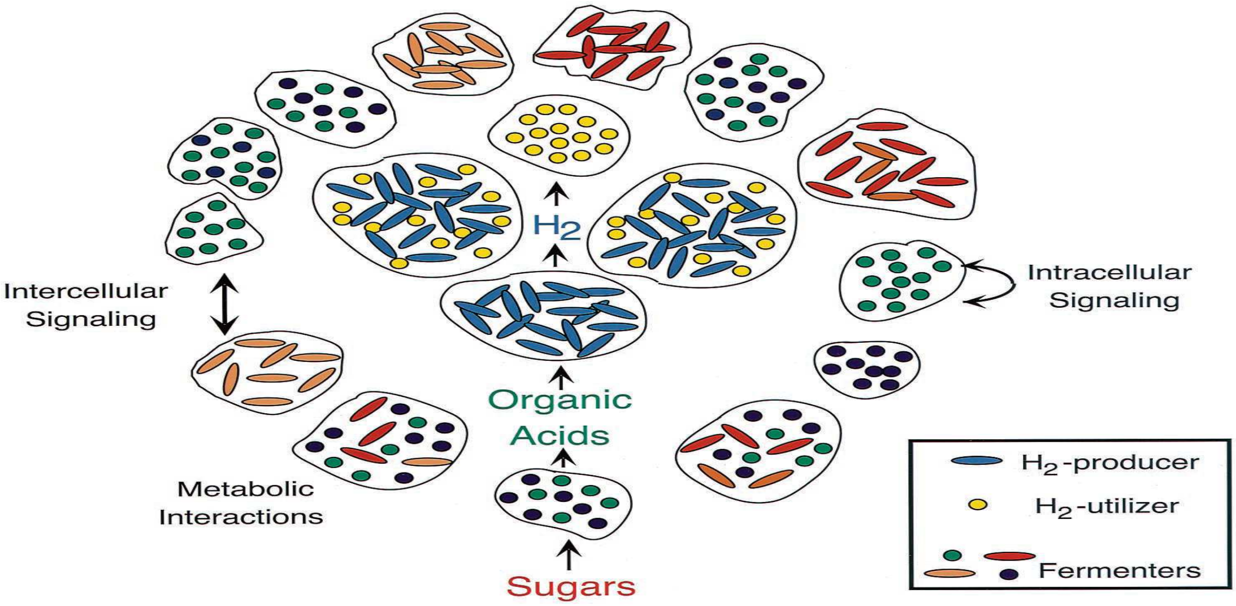


Figure 3: Ecology of microbial communities (Davey and O' Toole, 2000).

Figure 3 depicts an idealized surface-attached microbial community (Davey and O' Toole, 2000). Within the microbial community, microcolonies exist. These comprise of single or multiple bacteria species. The proximity of different microbes allows the possibility of physical interactions in addition to communication via diffusible factors. Signaling molecules aid in inter- and intraspecies communication (Davey and O' Toole, 2000). The spatial organization of the biofilm is dependent on the metabolic interactions between the organisms. On the outer layer of the biofilm are fermenting bacteria. These gain their carbon and energy by utilizing various sugars to produce organic acids that serve as substrates used by the hydrogen producing bacteria (Lin and Chang, 1999; Davey and O' Toole, 2000; Nath and Das, 2004). As illustrated in Figure 3, there are four microcolonies at the center of the figure representing organisms that both generate and consume hydrogen. These two organisms participate in syntrophism (Davey and O' Toole, 2000). The fermentation of organic matter can produce different end product, however, several metabolites such as acetic acid/acetate and butyric acid/butyrate are associated with anaerobic H_2 fermentation of carbohydrates. These have theoretical hydrogen values produced via the acetic acid and butyric acid pathways being 4mol and 2mol, respectively (Chang *et al*., 2008). In contrast propionic acid is considered an undesirable metabolite during fermentation as it inhibits growth of biohydrogen producing bacteria. These metabolites are collectively known as volatile fatty acids (VFA). These should be monitored during the fermentation process as they play a major role in metabolic shifting in the system from acidogenesis, H_2 production, to solventogenesis, production of ethanol, butanol, propanol or acetone (Levin *et al*., 2004). Should the major component of the VFA be acetic acid, then a high efficiency of hydrogen production would be observed.

1. 5 **Bacteria identified in studies for biohydrogen production**

To design efficient biohydrogen producing systems requires an understanding of the microbial population responsible for hydrogen production (Hung (a) *et al*., 2011). Several authors have stated that hydrogen production is influenced by metabolic pathway shift within the bacterial community in response to environmental conditions (Levin *et al*., 2004; Karadag and Puhakka, 2010; Hung (a) *et al*., 2011). Several studies have been done on the environmental conditions that affect biohydrogen production (Levin *et al*., 2004; Gaval *et al*., 2006; Akoa *et al*., 2007; Masilela and Gray, 2008; Wu *et al.,* 2008; Karadag and Puhakka, 2010; Lay *et al*., 2010; Hung (a) *et al*., 2011; Ngoma *et al*.,2011). These are temperature, pH, nutrient source and the hydraulic retention time within a bioreactor. These factors influence the type of microorganisms that would be present in the bioreactor, therefore influencing hydrogen production.

Anaerobic biohydrogen production has been reported at temperatures ranging from 25°C to 80°C (Levine et al., 2004; Karadag and Puhakka, 2010). Yu *et al.* (2002) established that H_2 production increased with increasing temperature. Gavala *et al.* (2006) found that high H_2 production at 55°C was associated with a decreased biomass while Lin *et al.* (2008) found that changes in H_2 production at different temperatures was associated with a shifting in the metabolic pathways of hydrogen production (Gavala *et al.*, 2006; Lin *et al.*, 2008). Karadag and Puhakka (2010) found that hydrogen production was high at 45°C and 60°C, each corresponding to a different metabolic pathway based on the dominant microorganism present in the bioreactor. At 45°C, the metabolic pathway was a butyrate type fermentation with *Clostridium* spp. dominant in the bioreactor, while at temperatures 45- 55°C, hydrogen production was low with the dominant microorganism being *Bacillus coagulans* (Karadag and Puhakka, 2010). At 60°C the dominant bacteria was *Thermoanaerobacterium* spp. and the methabolic pathway was an ethanol type of fermentation, resulting in hydrogen production which was a third of the hydrogen produced at 45°C (Zhang *et al.*, 2003; Georgi *et al.*, 2005; Wang *et al.*, 2007).

The HRT controls the microbial growth of rate of microorganisms (Hung (a) *et al.*, 2011). Wei *et al.* (2010) found that low HRT have an adverse effect on bioaugmentation as it leads to biomass washout and for bioaugmentation. It was found that the HRT should not be less than 6 h (Wei *et al.*, 2010). Lay et al. (2010) observed a microbial community shift from *Clostridium* spp. to *Acidaminococcus* spp when the HRT as reduced and the major VFA shifted from butyrate to acetate (Lay *et al.*, 2010). *Acidaminococcus* spp dominated the bioreactor and high concentrations of acetate were observed at HRT 3h.Whereas, *Clostridium* spp. dominated at HRT 12h, 8h, 6h and 4h with the emergence of *Acidaminococcus* spp at HRT 4h. The best hexose degradation was observed at HRT 3h, 4h and 6h, with HRT 4h having the optimum hexose degradation of 74.9% accredited to the presence on both the *Clostridium* spp and *Acidaminococcus* spp. Thus, Lay et al. (2010) suggested a co-cultivation of *Clostridium* spp with *Acidaminococcus* spp to improve substrate degradation. Lay et al. (2010) believed that the dominant *Acidaminococcus* spp degraded glutamate produced by *Corynebacterium* spp to produce hydrogen (Lay *et al.*, 2010). Butyrate yields were high at HRT 24h and HRT 12h, due to the dominance of *Clostridium* spp (Lay *et al.*, 2010). The longer HRT allows for the growth *Clostridium* spp. Luo *et al.* (2008) investigated the effects of the organic loading rate (OLR) on biohydrogen production and the bacteria community structure (Luo *et al.*, 2008). The authors found that *Clostridium* FRB1 was the most dominant when the OLR was 5, 7.5 and $10gl^{-1}$, while *Clostridium acetobutylicum* and *Clostridium acidisoli* were the dominant species when the OLR was 7.5g at an HRT of 10h. The same study found that *Selemonas* strain SB 90, *Enterobacter* sp B509, *C. acetobutylicum* *Clostridium magnum* and *Clostridium* FRB1 were the predominant species at $2.5gl^{-1}$ (Luo *et al*., 2008). It was also concluded that *Selemonas* spp contributed to greater biohydrogen production at low OLRs (Luo *et al*., 2008; Hung (a) *et al*., 2011). Hung (b) *et al.* (2011) investigated the interactions between *Clostridium* spp. with other bacterial species within a biohydrogen producing system and found that *Klebsiella* spp. was among the dominant species at HRT 6h to 1h (Hung (b) *et al*., 2011). *Klebsiella* spp. disappeared at HRT 0.5h and *Clostridium pasterianum* was the dominant hydrogen producer throughout the different period. *Streptococcus* emerged at HRT 2h and remained in the system together with *C. pasterianum* at HRT 0.5h (Hung (b) *et al*., 2011).

Other classes of bacteria that have been reported as biohydrogen producers are Thermoanarobacteriales, particularly species like *Thermosaccharolytic* and *T. lactoethyicum* (Zhang *et al*., 2003; Georgi *et al*., 2005; O-Thong *et al*., 2009; Karadag and Puhakka, 2010; Hung (a) *et al.,* 2011) and Veillonellacaea, particularly *Selemonas* spp, *Acidaminococcus* spp, *Megasphera* spp and *Mitsoukella* spp (Koskien *et al*., 2000; Hung (b) *et al*., 2011; Lay *et al*., 2010).The presence of Enterobacteria and facultative anaerobes in biohydrogen producing systems have been observed as a way to maintain strictly anaerobic conditions, thus promoting biohydrogen production (Hung (b) *et al*., 2011).

1.6 **Methods used for the analysis of biohydrogen- producing microbial communities**

Recent studies of biological hydrogen production have used indirect methods like metabolites distribution, enrichment methods, and microscope examination, molecular techniques like DGGE, DNA- cloning analysis, dot-blot hybridization, terminal restriction fragment length polymorphism in order to determine microbial species composition in hydrogenogenic processes (Valdez-Vazquez and Poggi-Varaldo, 2009).

Many biochemical, nutritional and physiological characterization tests have been established in bacterial systematics e.g. indole test, oxidation-fermentation, cytochrome- oxidase, catalase, amino peptidase and the KOH test (Busse *et al*., 1996). Usually these tests are carried out in solid and/or liquid media, however, only a small percentage of bacteria are culturable (Busse *et al*., 1996). These tests are laborious, time- consuming and often difficult to standardize and interpret. Since the 1970's, when Carl R. Woese and co- workers began to investigate the phylogeny of prokaryotes by cataloguing 16SrRNA, knowledge in the phylogeny of prokaryotes has greatly increased (Busse *et al*., 1996). Using 16SrRNA they clearly demonstrated that life can be divided into three major lines of descent now named the domains Archae, Bacteria and Eucarya which are formerly known as archaebacteria, eubacteria and eukaryotes (Busse *et al*., 1996).

1.7 **Denaturing gradient gel electrophoresis DGGE**

Problems have been experienced when attempting to identify bacteria found in their natural habitat and in their natural community (Busse *et al*., 1996). These problems arise from bacteria requiring cultivation on growth media in order to amplify the number of cells. However, from a natural community only 0.001- 15% of the organisms can be cultured in comparison with total count methods (Busse *et al*., 1996). This is due to many microorganisms growing syntrophically with others , thus cannot be cultured individually (Fang(a) *et al*., 2002; Fang(b) *et al*., 2002). For this reason, this study has chosen PCR DGGE as the best method to use for biohydrogen producing bacteria identification.

For the detection of more members within a natural community, the development of the PCR techniques has solved some of the problems. DGGE involves running the PCR products obtained from the natural sample are run on a polyacrylamide gel with a gradient of two denaturants (Urea and Formamide) (Busse *et al*., 1996). The separation of the mixture of DNA fragments of the same length is based on electrophoretic mobility of partially melted DNA molecules of differing base composition (Busse *et al*., 1996). The melting behaviour of DNA fragments is highly dependent on the DNA sequence, therefore a single nucleotide difference would ensure that the DNA melts at different point on the gradient gel (Hayes *et al*., 1999). Muyzer *et al*. (1993) found that incorporation of a GC clamp into one of the primers used for PCR modified the melting behaviour of the fragments of interest, making it possible for almost all sequence variants to be detected. DGGE allows for a qualitative and a semiquantative analysis of a microbial community by indicating the presence and relative abundance of the different species within the sample (Muyzer *et al.,* 1993). The DGGE approach is advantageous over the cultivation strategy or analysis of community structures as more members of the community can be detected simultaneously (Busse *et al*., 1996). However, it cannot be assumed that all strains of a given sample are amplified.

For optimal hydrogen production, a thorough knowledge of the ecology of the hydrogen-producing microbial community which is enriched from environmental samples is required in addition to optimizing reactor design, in order to reveal factors influencing the efficacy and stability and to develop promising strategies for improved process performance (Hung *et al.*, 2007). In order to enhance the process and maintain an attractive hydrogen production, it is advisable to gain insight into the community structure and dynamics (Valdez-Vazquez and Poggi-Varaldo, 2009).

1.8 **Study objectives:**

- Develop an efficient protocol for bacterial identification through PCR- DGGE
- To analyse the microbial community responsible for biohydrogen production in the three thermophilic Anaerobic Fluidised Bed Bioreactors (AFBRs)
- To identify good candidates for bioaugmentation by obtain pure cultures of bacteria involved in hydrogen production.
- To determine bacterial morphology using scanning electron microscopy

CHAPTER 2: MATERIALS AND METHODS

Most studies that have identified biohydrogen producing bacteria, were undertaken at mesophyllic conditions. This study focuses on the microorganisms that are capable and involved in biohydrogen under thermophilic conditions using two different sources. Three basic methods were followed. The first bioreactor was kept at a constant temperature of 65°C with sewage used as a source for the potential biohydrogen producers. The second bioreactor had a temperature range from 45°C to 65°C with sewage as the microorganism source. For the third bioreactor, cow dung was used as a source for microorganisms which have the potential to produce biohydrogen. The temperature range was from 55°C to 70°C. The cow dung was used in the hope of isolating hydrogen producing bacteria that are also capable of degrading cellulose, as these would be ideal in the biodegradation of food processing effluents. With the increase in the human population, food processing activities are bound to increase that microorganisms that can generate an alternative fuel such as hydrogen using effluents with high cellulose content would be ideal for bioaugmentation of treatment plants for food processing wastewaters.

2.1. Bioreactor 1 Set up: Thermophilic bioreactor with sewage sludge as an inoculum

The experimental set-up (Figure 4) consists of an Anaerobic Fluidised Bed Reactor (AFBR) vessel made of cylindrical clear Perspex column surrounded by a second Perspex tube which forms the water jacket. The base of the bioreactor is lined with cylindrical activated carbon (CAC) particles ranging from 4 to 8 mm in length was used as a carrier substrate, an attachment material to induce bacterial attachment, and evenly packed at the bottom of the column reactor at 30% of its height. The bioreactor vessel is attached to a liquid-gas separator column. The effluent from the bioreactor will flow into a liquid-gas separator column which also has a water jacket. The liquid-gas separator column was designed to serve two functions; firstly for gas collection and as an effluent recycle reservoir. Two 520U Watson Mallow Bredel pumps (Falmouth, UK) were used; one to introduce the 3x concentrated Endo nutrient feed into the reactor column and the second pump used to dilute the 3x concentrated feed with water (Masilela and Gray, 2007, Ngoma *et al*., 2011). A Boyser® Bonfigloli pump (Barcelona, Spain) was used to recycle the effluent and the nutrient medium through the reactor vessel. The effluent outlet at the top of the reactor column will be connected to the gas-liquid separator; from which biogas production is measured using a Type TG1 gas meter (Ritter Inc., Germany). The gas composition was analysed using gas chromatography (Clarus

500 GC PerkinElmer). Argon was used as the carrier gas at a flow rate of 2.0ml min^{-1}. The excess effluent will be collected from the gas-liquid separator column by an outlet for the removal of the excess effluent from the reactor into the drain. The operational temperature was maintained by pumping water through the water jacket connected to a heat exchanger and the pH kept between pH 5.0-6.0 controlled automatically by feeding NaOH and HCl via respective peristaltic pumps (Masilela and Gray, 2007).

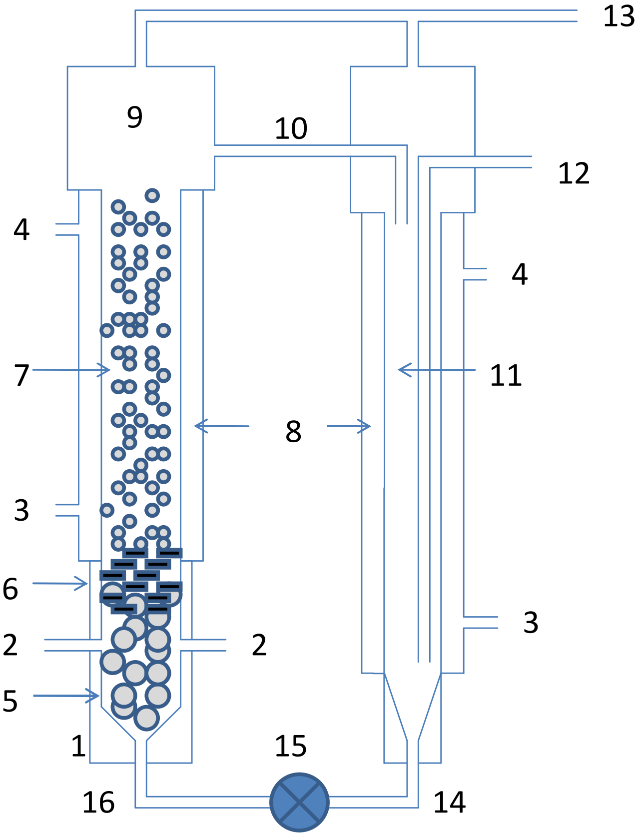


Figure 4: Schematic diagram of the anaerobic fluidised bed bioreactor used in the study. 1) Inlet manifold 2) Influent inlets 3) Water jacket inlet for heat exchanger 4) Water jacket outlet for heat exchanger 5) Glass beads bed (5mm) in effluent/influent diffusion and cavitation generation 6) Activated carbon for inducing granulation 7) Fluidised bacterial granular bed 8) Water jacket for heat exchanger 9) Effluent decanter 10) Effluent connecting pipe to gas disengager 11) Gas disengager tube 12) Effluent outlet overflow pipe 13) Gas flow pipe 14) Effluent recycle outlet pipe 15) Effluent recycle pump 16) Effluent recycle inlet (Ngoma *et al*., 2011)

The AFBR has a working volume of (approximately) 7.5L (Masilela and Gray, 2007). The reactor was run at 55 ºC for 8 days and then at a constant 65ºC for the rest of the experimental duration, maintained by pumping water through a water-jacket connected to a heat exchanger, at a pH of 5.5-6. The AFB reactor was run at an HRT of between 1-10 hours, with every HRT value applied for 4 days.

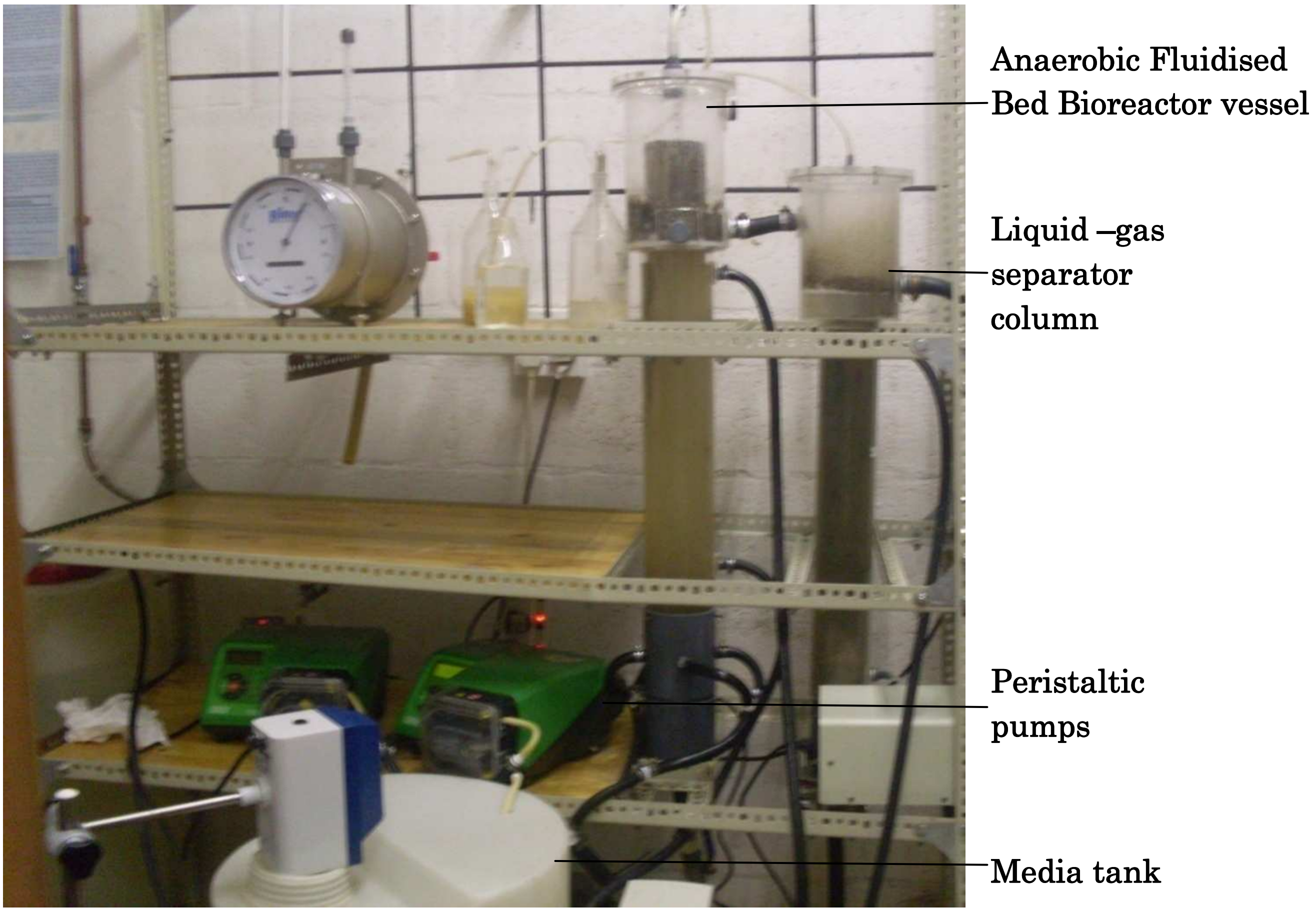


Figure 5: Bioreactor 1 experimental set up

The seed sludge was obtained from a local Olifantsvlei Waste Water treatment Works and Regional sludge handling facility in the South of Johannesburg, South Africa (Soweto). The collected sewage sludge was heated at 100 ºC for 45 h. Thereafter, the sludge was subjected to acid pre-treatment to inactivate methanogenic bacteria and acid-sensitive non-H_2-producing bacterial population in the sludge. The acidic pre-treatment involved a decrease in sludge pH to 2.0 by 0.1N HCl for 24 h and a readjustment of pH back to 7.0 by 0.1N NaOH.

The nutrient used for the continuous fermentation was obtained by diluting sucrose as limiting carbon substrate with non- sterile tap water until the desired sucrose concentration (17.5g/L) is achieved and by adding the following sufficient inorganic and mineral supplements (in g/L) (Endo et al., 1982) including: NH_4CO_3 3.49, $NaHCO_3$ 6.72, K_2HPO_4 0.7, $CaCl_2$ $2H_2O$ 0.2, $MgCl_2$ $6H_2O$ 0.1, $MnSO_4H_2O$ 0.015, Fe SO_4 $7H_2O$ 0.025, $CuSO_4$ $5H_2O$ 0.005, $CoCl_2$ $6H_2O$ 0.00018 (Saarchem®, Merck (Pty) Ltd, Wadeville, Johannesburg, South Africa). All chemical were obtained from Merck (Pty) Ltd, Wadeville, Johannesburg, South Africa.

2.2. Bioreactor 2 Set up: Thermophilic bioreactor with sewage sludge as an inoculum

The reactor is constructed in the same as Bioreactor 1, however, it had a working volume of 12 L. The reactor was run at 45ºC, 50ºC, 55 ºC, 60 ºC, 65ºC at 8 day intervals for each temperature maintained by pumping water through a water-jacket connected to a heat exchanger, at a pH of 5.5-6.

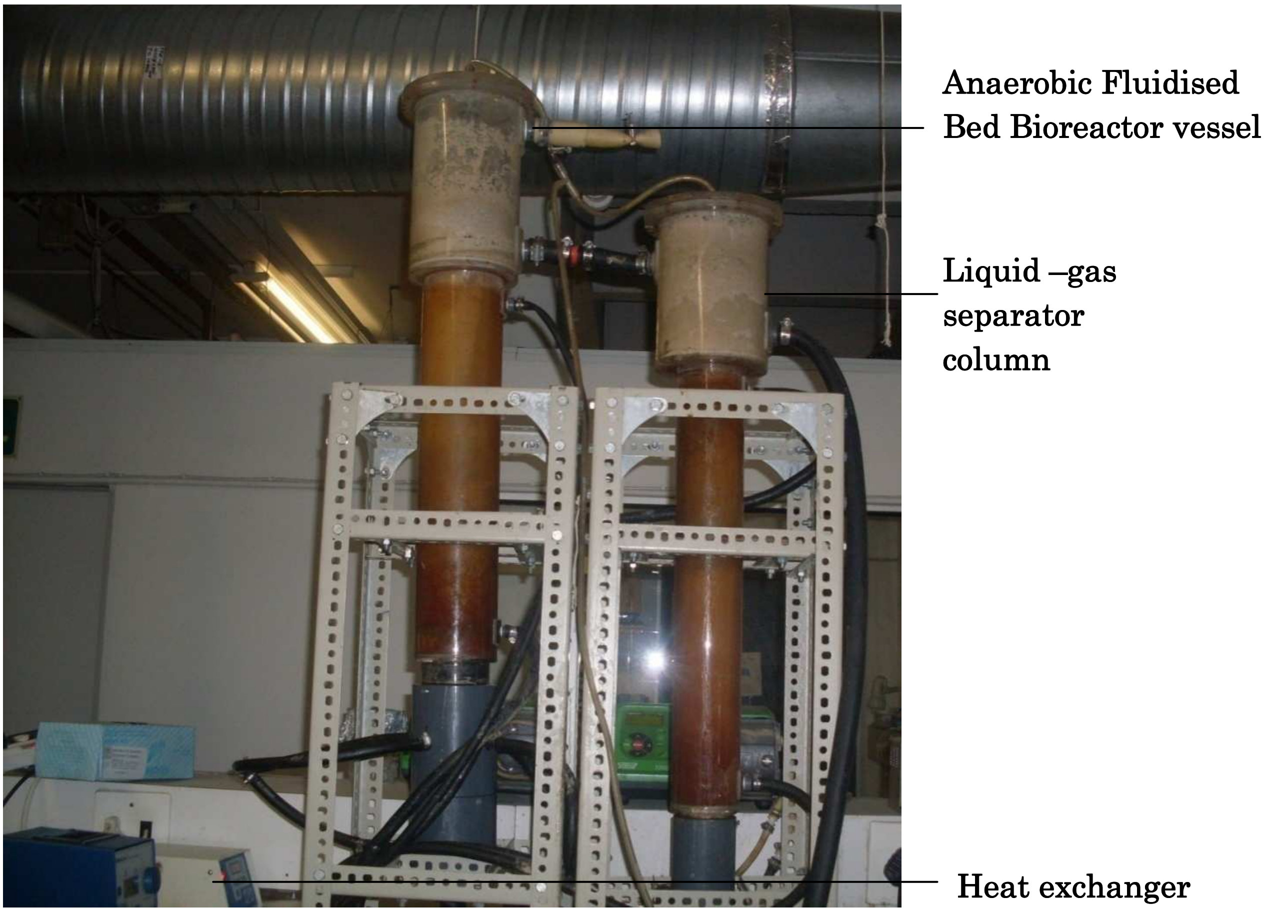


Figure 6: Bioreactor 2 experimental set up

The sample collection procedure and nutrient medium used are the same as those for the Thermophilic Bioreactor 1. The AFB reactor was running in batch mode for the initial 48 hours by adding treated seed sludge and feed with fresh modified Endo formulation. After 48h the reactor was changed to a continuous mode at HRT of 4 h to stimulate sludge granulation under anaerobic conditions. After reaching steady-state operation, the HRT was decreased to 2 h, at which self-flocculated sludge granulation occurs.

2.3. Bioreactor 3 Set up: Thermophilic bioreactor with cow dung as an inoculum

The reactor is constructed in the same as Bioreactor 1, however, it has a working volume of 18 L. The reactor was run at 55ºC, 60ºC, 65 ºC, 70 ºC at a pH of 5.5-6.

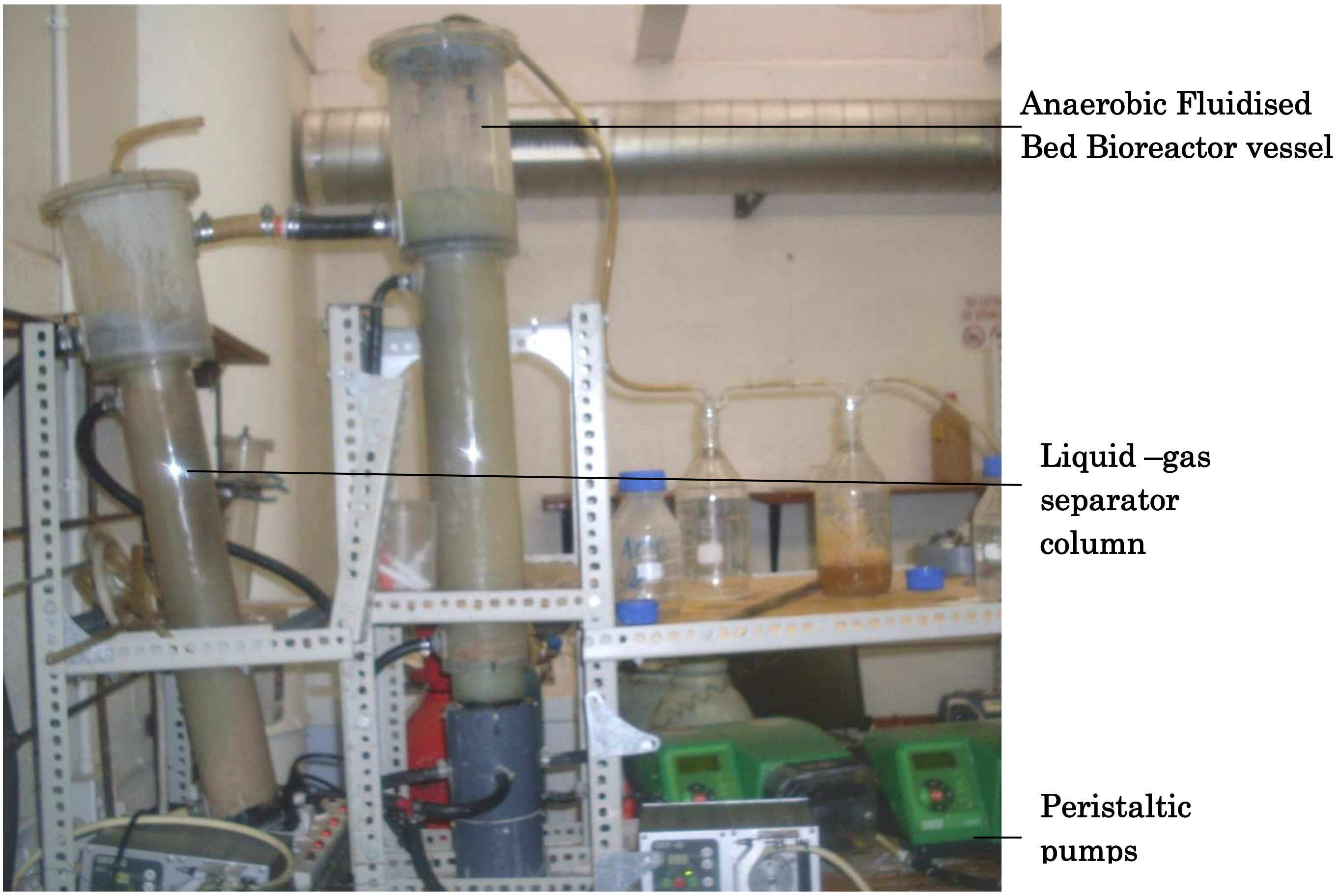


Figure 7: Bioreactor 3 experimental set up

The cow dung samples were obtained from the Animal and Dairy Science Research Institute (Irene, Pretoria, South Africa) and heated at 100 ºC for 45 h. Thereafter, it was subjected to acid pretreatment to inactivate methanogenic bacteria and acid-sensitive non-H_2-producing bacterial population in the sludge. The acidic pretreatment involved a decrease in sludge pH to 2.0 by 0.1N HCl for 24 h and a readjustment of pH back to 7.0 by 0.1N NaOH. The AFB reactor will be running in batch mode for the initial 48 hours by adding treated seed sludge and feed with fresh modified Endo formulation. After 48h the reactor was changed to continuous mode at HRT of 4 h to stimulate sludge granulation under anaerobic conditions. After reaching steady-state operation, the HRT will be decreased to 2 h, at which self-flocculated sludge granulation occurs.

The media used was *Clostridia* media made up of the following inorganic salts and the concentration; 2g of $FeSO_4.7H2O$, 2g of $MgSO_4.7H_2O$, 20g of $CaCl_2$, 20g of NH_4Cl, 20g of $Na_2HPO_4.2H_2O$, 40g of KH_2PO_4 (Saarchem®,Merck (Pty) Ltd, Wadeville, Johannesburg, South Africa), and 8g of yeast extract (Biolab®, Merck (Pty) Ltd, Wadeville, Johannesburg, South Africa),

All the bioreactors had an experimental duration of 40days.

2.4 **Bacterial identification**

2.4.1 **Pure culture isolation**

Liquid samples (15ml) were obtained from the AFBRs and centrifuged at 2200rpm in a bench centrifuge for 10 minutes. The pellet was resuspended in 1ml sterile distilled water. Samples were streaked on three types of media i.e. nutrient agar (Biolab®, Merck (Pty)Ltd, Wadeville, Johannesburg, South Africa), blood agar (Selecta-MEDIA, Strijdom Park, Johannesburg, South Africa) and Endo media as described in Section 2.1 with 15g Agar powder (Biolab®, Merck (Pty)Ltd, Wadeville, Johannesburg, South Africa), added to solidify the medium. These were incubated anaerobically, in an Oxoid 2.5L AnaeroJar ™ using the AnaeroGen ™ system (Oxoid Ltd, Basingstoke, Hampshire, England), at 55°C for 48 hours. Single colonies of different bacteria isolated from each plate are inoculated onto Luria Bertani agar, and incubated anaerobically, in a Oxoid 2.5L AnaeroJar ™ using the AnaeroGen ™ system (Oxoid Ltd, Basingstoke, Hampshire, England) at 55°C for 48 hours. Single colonies were inoculated into Luria Bertani broth with 2% Sodium Thioglycollate (Sigma®, Sigma- Aldrich (Pty) Ltd, Kempton Park, Johannesburg, South Africa), a reducing agent that creates anaerobic conditions when molecular oxygen is reduced to water, and incubated at 55°C for 48 hours. Luria Bertani broth consists of 10g Tryptone, 5g Yeast Extract (Biolab®, Merck (Pty) Ltd, Wadeville, Johannesburg, South Africa), 10g NaCl and 1g glucose (Saarchem®, Merck (Pty) Ltd, Wadeville, Johannesburg, South Africa) per Litre of medium. The Luria Bertani Agar was made up similarly to the LB broth with 15g of Agar powder (Merck (Pty)Ltd, Wadeville, Johannesburg, South Africa) added to solidify the medium.

2.4.2 **Morphology determination**

2.4.2.1 **Gram Staining**

Pure colonies obtained from the anaerobic plates were selected and smeared on a glass slide for the Gram staining procedure which was as follows; bacterial smears were air dried, then heat fixed by passing though a flame. Once heat fixed, the smears were flooded with Crystal Violet (0.5% crystal violet solution, Saarchem®, Merck (Pty) Ltd, Wadeville, South Africa) for 1 minute. This was followed by gently rinsing off the stain with water and flooding with Gram's Iodine (20g Potassium Iodine and 10g Iodine per Litre, Saarchem®, Merck, Wadeville, South Africa) for 1 minute. After which, the iodine was gently rinsed off the stain with water. The smear was then flooded with the decolouriser made up of Acetone (70% v/v) and 30% (v/v) Ethanol (Saarchem®, Merck(Pty)Ltd, Wadeville, South Africa) for approximately 10-20 seconds. As soon as colour was seen running off the slide, the smear was rinsed off with water and flooded with the counter stain, Safranin (1% Safranine O solution, Saarchem®, Merck (Pty) Ltd, Wadeville, South Africa) for 30 seconds. Lastly, the slides were rinsed off with water and blotted dry.

The gram stained bacterial smears were observed under oil immersion, using Olympus X21 light microscope and photographed using DCE-2 Digital camera eyepiece (Gentaur Molecular Products, Brussels, Belgium) operated using the PhotoScope ™software (ScopeTek® Opto-Electric Co., Ltd, /Hangzhou, Zhejiang Province, China).

2.4.2.2 **Scanning Electron Microscopy**

Samples of granules formed in the AFBRs were removed from the reactor using a sterile spatula and rinsed three times to remove unattached cells. The granules were fixed overnight at room temperature in 3% Gluteraldehyde (Saarchem®, Merck (Pty)Ltd, Wadeville, Johannesburg, South Africa) (Masilela and Gray, 2007). The granules were dehydrated using graded ethanol in the following concentrations 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and finally 100% for 10 minutes each. The samples were critically dry mounted and coated with gold- palladium. The samples were viewed under the JSM-840 scanning electron microscope (Masilela and Gray, 2007).

2.5 **DNA Extraction**

Duplicate samples of AFBR liquid samples suspended with granules were removed and stored at - 20ºC. Before DNA extraction of the AFBR liquid samples, cells were pelleted by centrifugation (10min at 10 000g) the supernatant discarded, then resuspended in 200µl of sterile distilled water (Koskinen *et al*., 2007) and the bacterial genomic DNA from the attached granules in the reactor was extracted using the Zymo Research ZR Fungal/ Bacterial DNA kit ™, according to the manufacturer's instructions (Inqaba Biotechnical Industries, South Africa).

2.6 **Polymerase Chain Reaction (PCR)**

Polymerase chain reaction was used to amplify the bacterial DNA extracted from samples isolated from the AFBRs and again to amplify the DNA isolated from the gel bands, as each band represents a different bacterial organism.

The primer sets used for the amplification of 16S rDNA were UNIV1392R: 5'-ACG GGC GGT GTG TRC-3', EUB968F AAC GCG AAG AAC CTT AC and EUB968F with GC clump in combination with 2X PCR Master mix (*Taq* DNA polymerase (recombinant) in reaction buffer, $MgCl_2$ and dNTPs 0.4 mM of each. An automated thermal cycler (Applied Biosystems GeneAmp® PCR System 2700, USA) was used for PCR amplification, using the following program: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation (35 sec at 92 °C), annealing (1 min at 55 ° and extension (1 min at 72 °C), and a final extension at 72 °C for 5 min before storage at 4 °C (Masilela and Gray, 2007). To confirm the presents of amplified DNA, the PCR-amplified product were loaded and ran on 1 % (m/v) agarose gel. The MassRuler ™ DNA ladder, Low range (Fermentas, USA) was used as the molecular marker (Masilela and Gray, 2007).

2.7 **Denaturing gradient gel electrophoresis (DGGE)**

DGGE of the amplified PCR products were performed using a DCode Universal Mutation Detection System (Bio-Rad Laboratories, California., USA). The PCR products were applied to 6 % (w/v) polyacrylamide gel one containing a 20%-60 % gradient of denaturant and the other a 20%-70% (100% denaturant was defined as 7.0M urea and 40% deionized formamide). The electrophoresis was run in a 0.5X Tris-acetate-EDTA (TAE) buffer solution at various constant voltages of 100V, 120V, 130V, 160Vor 200V (These were the most commonly used voltages in literature) and 60 ºC or 65°C for 5 hours. After electrophoresis, the gels were stained for 15 minutes in 250 ml of 0.5X TAE buffer containing 10 µl of a 10 $mg.ml^{-1}$ ethidium bromide. Subsequently, the stained gels were washed for another 15 minutes in 250 ml of 0.5X TAE buffer. The gels were removed from the destaining solution and immediately visualized in the Gel Doc. Thereafter the dominant bands were excised from the gels under UV visualization. The bands on the gels were excised using a sterile razor blade for each band. The DNA elution step was carried out in three basic methods to determine the best method for elution. In the first method gel slices were eluted overnight in 20µl sterile distilled water in 1.5ml centrifuge tubes and boiled at 55°C. In the second method the gel bands were eluted 25µl sterile distilled water at 4°C overnight. The last method the gel slices were placed in 100µl of TE buffer overnight at 4ºC. The tubes containing the gel slices were centrifuged at 7000rpm for 30 seconds and aliquots of the supernatant used for re-amplification of the eluted DNA. Re-amplified of the DNA samples is as described in Section 2.6 with exception that forward primer which does not have a GC-clamp. After re-amplification under the reaction conditions described in Section 2.6, the DNA samples were sent for sequencing (Masilela and Gray, 2007).

2.8 **Sequence determination and bacterial identification**

The DNA (25µL) isolated from the gel slices as described in Section 2.7 was aliquoted into 50µL PCR tubes and the samples sent on dry ice to Inqaba Biotechnical Laboratories, South Africa and the University Stellenbosch DNA sequencing unit, South Africa for DNA sequencing. The sequences obtained from the sequencing companies for each sample were edited using Finch TV version 1.4.0 software. The resultant sequences were converted to Fasta format and uploaded to the Ribosomal Database Project archives. The uploaded sequences ware analysed using the RDP Naïve Bayesian Classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp) against16S rDNA sequences from the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu). The obtained DNA sequences and their closest 16S rDNA sequences of reference microorganisms retrieved from the RDP were used to construct rooted phylogenetic trees using the Weighbor weighted neighbour-joining tree building algorithm with 100 times bootstrapping. The phylogenetic trees were edited using Mega 5 software (2011). Sequence similarity was evaluated using DNAMAN version 4.0 software (Lynnon Biosoft, Department of Microbiology, University of Cape Town) comparing the obtained DNA sequences with the sequences of the closest reference microorganisms obtained from the National Centre for Biotechnology Information (NCBI) Genbank database.

CHAPTER 3: RESULTS AND DISCUSSION

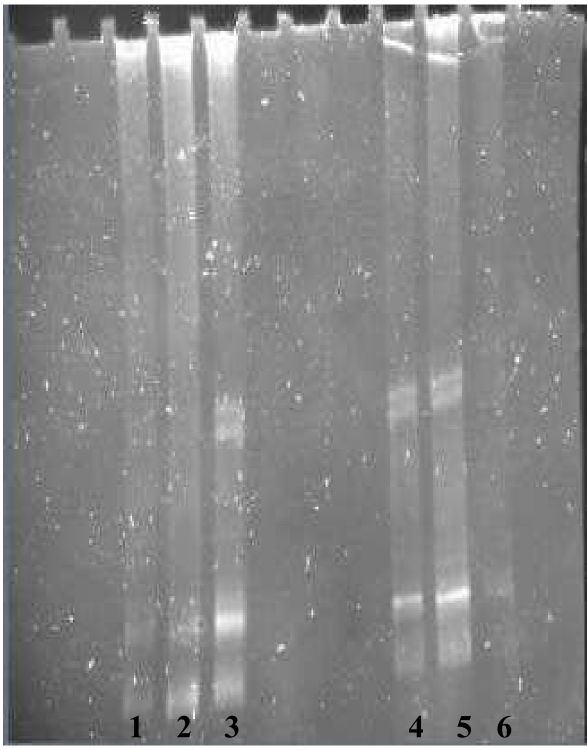
3.1 Optimization of PCR-DGGE procedure

The success in DNA profiling of the microbial community using DGGE is dependent on the melting profile of the DNA molecule applied to the gel and the gel system used (Hayes *et al.*, 1999). Prior to this study our research team had not been successful in isolating DNA from the DGGE gel slices. The team had established that a 6% agarose gel with a gradient of 20-60% was the best for separation of different strains of bacteria. The DNA elution was done in 25µl sterile distilled water overnight boiled at 55°C. Hayes *et al.* (1999) found that the 6% agarose gel allowed for faster migration of the DNA bands, thus allowing for optimal band separation (Hayes *et al.*, 1999). In the initial stages of this study it was observed that DNA bands which were close together could not be excised successfully as they appeared as a single band on the UV transilluminator. Since the isolation of DNA is crucial for identification of microorganisms responsible for biohydrogen production, this study undertook to optimise the PCR-DGGE process for the identification of microorganisms responsible for biohydrogen production at thermophilic temperature. The identification would aid in enhancing bioreactor design for optimum biohydrogen yields. Hayes *et al.* (1999) discovered that optimal DGGE band separation is influenced by several factors including voltage, the buffer used and the buffer temperature (Hayes *et al.*, 1999). In this study the buffer temperature, the gel gradient and voltage were investigated by comparing buffer temperature of 60°C and 65°C. Similarly applied voltages of 100V, 130V, 160V and 200V will be compared. Likewise, gel gradients of 20-60% and 20-70% were compared.

Of the two temperatures most frequently used in literature (60°C and 65°C), the 60°C gel running temperature was used more (Ahn *et al.*, 2005; Koskinen *et al.*, 2000; Nicolaisen and Ramsing, 2002; Hung *et al.*, 2007; Borole *et al.*, 2009; O-Thong *et al.*, 2009) followed by gels ran at 65°C (Maintiguer *et al.*, 2008). Both these temperatures were investigated for all the optimization steps (Data not shown) and the gels ran at 65°C were resulted in better DNA band resolution, therefore, the results shown below are for gels ran in 1x TAE buffer at 65°C. Hayes *et al.* (1999) found that DNA bands became fuzzy when high voltages were applied to a gel at 60°C and that to resolve this issue increasing the buffer temperature to 65°C improved resolution (Hayes *et al.*, 1999). Since 1x TAE was used as the running buffer a higher temperature of 65°C proved to be beneficial in overcoming the high resistance in the system caused by a high ion concentration in the buffer (Hayes *et al.*, 1999).

3.1.1 Gradient determination

Literature was investigated in order to determine an experimental gradient which will present better DNA band resolution than the established 20-60% gradient. Ueno et al. (2001) used a gradient of 20-60% to determine the microbial diversity of biohydrogen- producing community obtained from sludge compost (Ueno *et al*., 2001). Thornhill *et al*. (2010) investigated the effect different gradients had on the migration of amplicons using the BioRad DCode^{TM} system compared to the C.B.S Scientific^{TM} system and found that different sequences could not be separated on a gel with a gradient of 45-80% (Thornhill *et al*., 2010). The authors also found that distinct bands were present on gels with a gradient of 25-55% and 30-60% (Thornhill *et al*., 2010). Maintiguer *et al*. (2008) used a gradient of 30-60% and to investigate a hydrogen producing microbial consortium while, O- Thong *et al*. (2009) used the same gradient to investigate how seed inocula preparation affected biohydrogen production and the microbial community involved. Ahn *et al*. (2005) used a gradient of 40-60% to determine the microbial community involved in hydrogen production on a biofilter (Ahn *et al*., 2005). Hung et al. (2007) used a gradient of 30-65% when doing a quantitative analysis of the hydrogen producing microbial community in an agitated sludge bed bioreactor (Hung et al., 2007). Borole *et al*. (2009) used a gradient of 35-65% to investigate the microbial community in an acetate-fed microbial fuel cell (Borole *et al*., 2009). Nicolaisen and Ramsing (2002) used a gradient 30-70% when studying the diversity of ammonium-oxidizing bacteria (Nicolaisen and Ramsing, 2002). Wang *et al*. (2007) used a gradient of 40-70% to analyse the microbial community obtained from activated sludge (Wang *et al*., 2007). Sekiguchi *et al*. (2002) used the 20-20% gradient to determine the bacterial community structure along the Changjang river (Sekiguchi *et al*., 2002). The test gradient 20-70% covers the full range of gradients observed in the literature and that previously determined by our research team. To determine the best gradient range of bacterial species separation samples were run on gels with gradient 20-60% (Figure 8) and 20-70% (Figure 9).



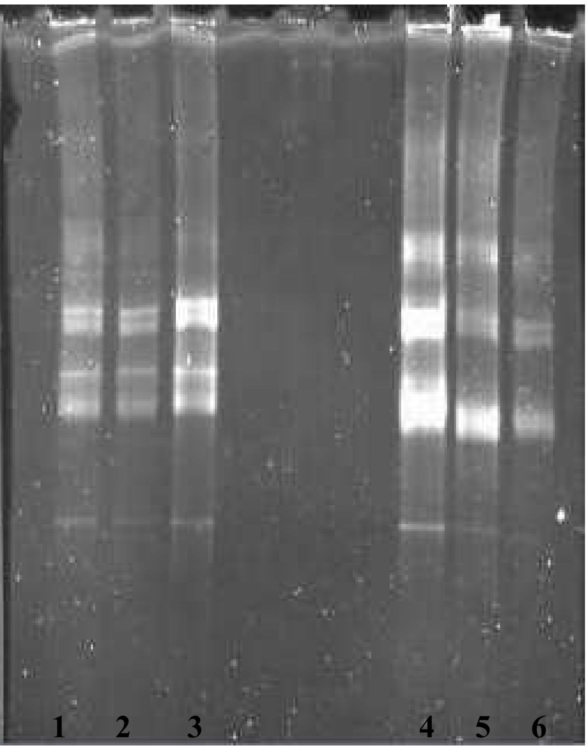


Figure 8: Bacterial DNA profiling on a 20-60% gradient gel

Figure 9: Bacterial DNA profiling on a 20-70% gradient gel

Lane 1,2,3 represent Bioreactor 1 samples, where the HRT was 7h,9h,and 10h at a constant temperature of 65°C, respectively, and Lane 4,5,6 represent Bioreactor 2 samples, where the operational temperature was 55°C, 60°C and 65°C at a constant HRT of 2h, respectively (Figure 8 and 9).

Gels with a gradient 20-60% produced bands that appeared faint whereas the same samples loaded on a 20-70% gradient appeared well defined. Gradient gels with a gradient of 20-70% (Figure 9) resulted in sharper band resolution, thus better separation of DNA bands for the bacterial strains. This gradient also resulted in a firmer gel than was the case when the gel has a gradient of 20-60% (Figure 8), thus making it easier to excise bands and the excised gel pieces were easier to handle for further processing. Since gels with the 20-70% gradient showed the best DNA band resolution, the DNA profiling results shown in Section 3.2 are of gels with a 20-70% gradient. Hayes *et al*. (1999) observed that band resolution deteriorated with smaller gradients and a gradient difference of between 30-50% between the low and high denaturant gave optimal band resolution. The 20-70% gradient had a gradient difference of 50%, therefore ensuring optimal band resolution.

3.1.2. Applied voltage

According to literature the most commonly used voltages were 100V (Koskinen et al., 2000; Nicolaisen and Ramsing, 2002; Thornhill *et al.*, 2010), 120V (Koskinen et al., 2000; Borole et al., 2009) and 200V (Ueno *et al.*, 2001; Nicolaisen and Ramsing, 2002; Ahn et al., 2005). Hayes *et al.*, (1999) tested the effect of voltage on DGGE profile for a broad range mutation analysis. They tested 50V, 100V, 150V and 200V on 9% agarose gel with a 25-65% gradient (Hayes *et al.*, 1999). It was observed that the amplicons reached the optimal melting temperature quicker with increasing voltage. Insufficient band resolution was seen at 50V and 100V when a 1x TAE buffer was used at a constant temperature of 59°C (Hayes *et al.*, 1999). A voltage of 150 showed better DNA resolution, however the intensity of the bands was higher when the potential applied to the system was 200V (Hayes *et al.*, 1999). Thornhill *et al.* (2010) investigated the effect voltage has on amplicon migration in the BioRad DCode^{TM} system compared to the C.B.S Scientific^{TM} system (Thornhill *et al.*, 2010). The study compared voltage 100V for 14h, 130V for 5h and 150V for 10h and found that 130V for 5h and 100V for 14h resulted in distinct dominant bands, where 150V resulted in smeared bands (Thornhill *et al.*, 2010). Moreover, it was found that the size of the gel influenced the running duration and the voltage that should be applied. The running duration is defined as voltage time. It appeared the shorter16cm gel for the BioRad DCode^{TM} system required a running time of 650Vh whereas, the 20cm gel for the C.B.S Scientific^{TM} system required 1400Vh for amplicon clear and distinct amplicon separation (Thornhill *et al.*, 2010). The BioRad DCode^{TM} system had the best results with distinct bands with a high intensity for gels with a gradient of 25-55% run at 130V for 5h and 30-60% gradient run at 100V for 14h than the gels run in the C.B.S Scientific^{TM} system (Thornhill *et al.*, 2010).

Previous work done in our research group had used 130V as the standard voltage applied to all DGGE analyses. In this study 100V, 120V, 130V, 160V and 200V were analysed, however results for 130V, 160V and 200V will be discussed. These potentials were applied to the gradient gels to determine the optimum potential for superior bacterial 16S rDNA fragment separation.

Six identical samples were loaded on gels with a 20-70% gradient and ran at different voltage 130V, 160V and 200V in a 1x TAE buffer at 65°C for 5 hours.

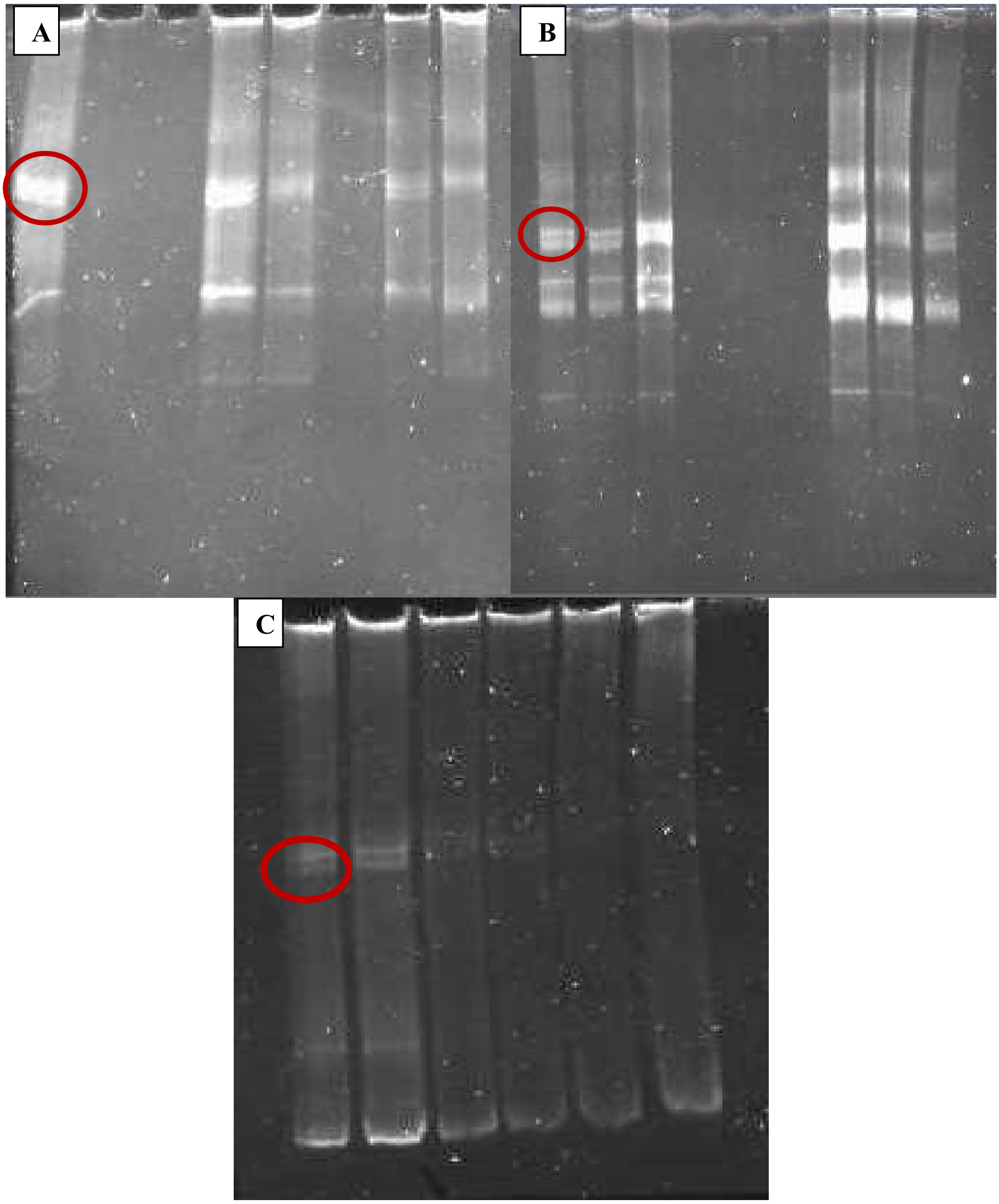


Figure 10: DGGE gels exposed to a potential of (A) 130V, (B) 160V and(C) 200V Denaturing gel electrophoresis was conducted at various voltages for 4 hours.

The gel run at 130V (Figure 10A) failed to resolve amplified DNA fragments which were similar causing a one large band instead of two separate bands as is the case for the gel ran 160V (Figure 10B) as illustrated by the bands within the red circle. The same band appeared faint on the gel run at 200V (Figure 10C), this be that the high voltage has caused some of the smaller DNA fragments to run off the gel.

The gels subjected to a potential of 160V had better band resolution in Figure 10B than those subjected to a potential of 130V and 200V depicted in Figure 10A and 10C, respectively. Thus a potential of 160V was applied to the gels used for microbial community identification in Section 3.2.

3.1.3 DNA Isolation step

Our research team had no success prior to this study in isolating DNA from gel slices. During the literature review three methods became appealing. Maintinguer *et al.* (2008) put the excised DNA gel bands in 20µl ultra pure water for 24hours in order to elute the DNA (Maintiguer *et al.*, 2008). Koskien *et al.* (2007) placed dominant gel slices in 25µL of sterile distilled water overnight at 4°C (Koskien *et al.*, 2007). Sekiguchi *et al.* (2001) and Sekiguchi *et al.* (2002) placed the excised gel slices in 100µl TE overnight at 4°C, while Xi *et al.* (2008) placed the gel slices in 50µl TE overnight at 4°C (Sekiguchi *et al.*, 2001; Xi *et al.*, 2008). In this study three methods were investigated firstly the gel slices were eluted in 20µl sterile distilled boiled overnight at 55°C (Results not shown). Secondly the gel bands were eluted 25µl sterile distilled water at 4°C overnight. Lastly the gel slices were placed in 100µl of TE buffer overnight at 4ºC.

Figure 11: 16S rDNA fragments eluted in distilled water

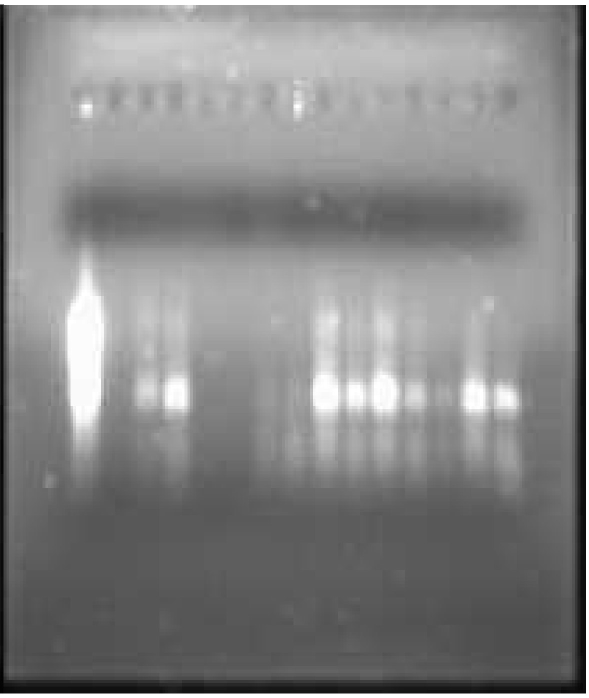


Figure 12: 16S rDNA fragments eluted in TE buffer

Gel slices placed in TE buffer (Figure 12) experienced enhanced DNA elution that those placed in distilled water (Figure 11) overnight at 4°C. The TE buffer preserved the integrity of the DNA strands and allowed for enhanced elution of the DNA from the gel slices.

The evaluated parameters, gel gradient, voltage and TE buffer elution, allowed for improved DNA isolation. The conditions for the DGGE procedure determined through the procedure optimization step were applied in the DGGE analysis of the 16S rDNA fragments obtained from the three hydrogen producing Anaerobic Fluidised Bed Bioreactors.

3.2 Bacterial Identification

The AFBRs had various operational temperatures, however, only two temperature points were of interest namely 55°C and 65°C, with 65°C being the preferential operational temperature. It has been demonstrated that thermophilic dark fermentation hydrogen production gives a higher reaction rate and better process performance as it results in increased energy recovery and decreased problems with contaminating hydrogen- consuming microorganisms (O-Thong *et al*., 2009; Ngoma *et al* 2011).

Anaerobic fermentation of substrates is usually carried out by various populations of bacteria (O-Thong *et al*., 2009). The most fermentative bacteria in hydrogen production include species of *Enterobacter*, *Bacillus* and *Clostridium* (Maintinguer *et al*., 2008; Hallenbeck and Ghosh 2010). This diverse microbiota ferments the sucrose within 30 hours (Combet-Blanc *et al*., 1995). This fermentation leads to the acidification of the medium through the production of organic acids (Combet-Blanc *et al*., 1995). The decrease in pH results in the selection of acidophilic species and the disappearance of Gram-negative bacteria, as well as pathogenic bacteria that cannot adapt to the low pH values (Combet-Blanc *et al*., 1995). Despite the selectivity of this ecosystem, some *Bacillus* and *Clostridium* spp. survive in this environment, probably because of their ability to sporulate (Combet-Blanc *et al*., 1995). Rod- shaped bacteria are predominant on the surface of the hydrogen producing granules as revealed scanning electron microscopy (SEM) and Gram staining images (Zhang *et al*., 2008). Culturable bacteria were isolated on three types of solid media and revealed two types of colonies were observed, however DNA analysis revealed that the two colonies were strains of the same species. The pure colonies were used to make smears for Gram staining and to inoculate the liquid media. The bacteria were cultured in liquid media in order to be used for bioreactor enrichment and DNA extraction.

3.2.1. Bioreactor One

3.2.1.1. Gram Staining

The culturable bacteria, in samples taken at 55°C, revealed rod-shaped Gram- positive bacteria (Figure 8). Single colonies were then inoculated into the liquid medium, Luria Bertani broth with 2% Sodium thioglycollate (Sigma®, Sigma- Aldrich (Pty) Ltd, Kempton Park, Johannesburg, South Africa), in order to grow enough cells for DNA extraction.

Figure 13: Gram stain of Bioreactor 1 culturable bacteria with the internal temperature of the bioreactor at 55°C

Molecular analysis of the 16S rDNA of the bacterial DNA identified that the culturable bacteria were *Bacillus coagulans*. *B. coagulans* is a Gram- positive, acid- tolerant and heat resistant facultative anaerobe (Kotay and Das, 2007). Its optimum growth temperature is 50°C, however it can tolerate temperatures of between 30°C- 55°C. It has the highest hydrogen yields when sucrose is the substrate yielding 3molH_2/ mol sugar in comparison to other substrates such as maltose, glucose and starch at low pH values of between 4.0 and 4.5 (Kotay and Das, 2007).

The culturable bacteria isolated when the operational temperature was 65°C, were Gram-negative rod- shaped bacteria with terminal and subterminal endospore, labelled A (Figure 14 and 15). Label B show the spores formed by the bacteria. This Gram- stain smear was obtained from a single colony on the nutrient agar (Biolab®, Merck (Pty)Ltd, Wadeville, Johannesburg, South Africa).

Figure 14: Gram stain of Bioreactor 1 culturable bacteria colony with the internal temperature of the bioreactor at 65°C

Molecular identification of the 16S rDNA of these bacteria identified them as *Clostridium thermopalmarium*. *C. thermopalmarium* is a Gram- positive, thermophilic, obligate anaerobic spore forming rod shaped bacteria (Soh *et al*., 1992). However, the gram stained cells appear to be Gram- negative as illustrated by Figure 14 and 15. Previously Soh *et al*., (1992) found that the *C. thermopalmarium* cells stained Gram- negative even in the early growth phase even though they possessed a cell wall profile of a Gram- positive bacterium (Soh *et al*., 1992).

Single colonies were then inoculated into the liquid medium in order to grow enough cells for DNA extraction.

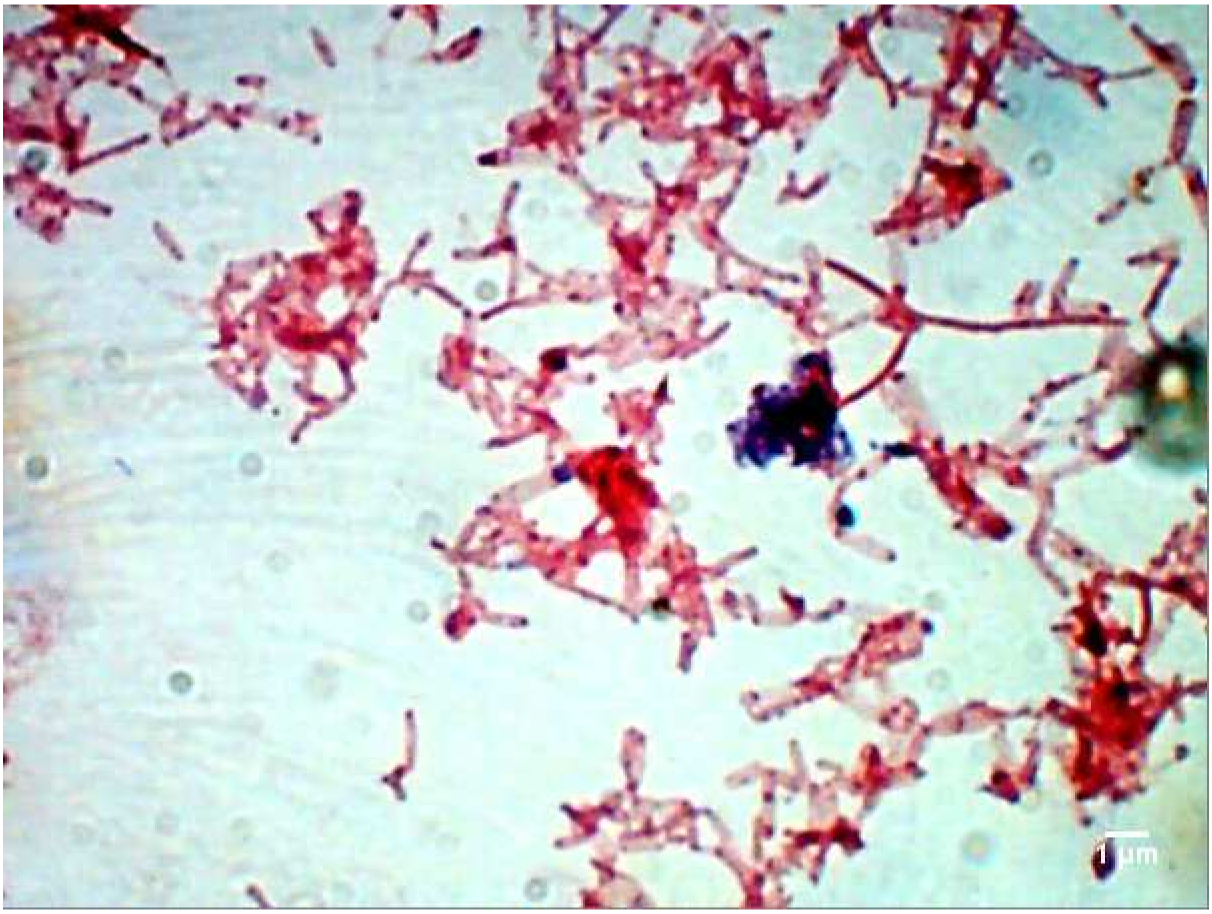


Figure 15: Gram stain of Bioreactor 1 culturable bacteria in liquid medium with the internal temperature of the bioreactor at 65°C

The *C. thermopalmarium* cells within the liquid media appeared to clump together (Figure 15), indicating that this species has the potential to self- granulate within the bioreactor. It was also observed that only cells with subterminal spores were present in the liquid culture and without extracellular spores (Figure 15).

3.2.1.2. Scanning Electron Microscopy

The granules used for the scanning electron microscopy preparations were taken when the operational temperature was at 65°C, as these granules proved to be more robust that those removed from the bioreactor at 55°C as they could withstand the SEM sample preparation without being crushed to fine powder.

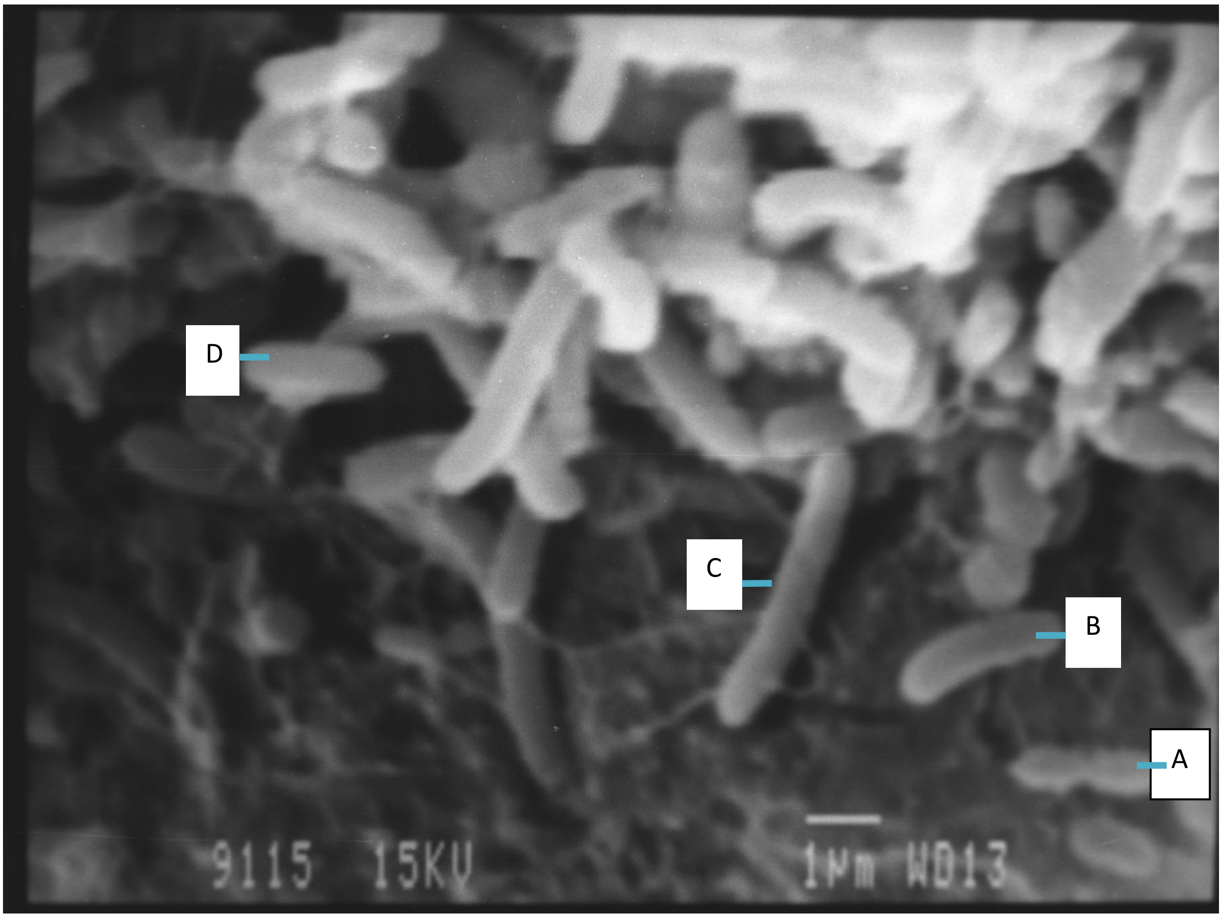


Figure 16: SEM micrograph of Bioreactor 1 granule depicting A) diplococcal bacteria B) slightly curved rod shaped bacteria C) long rod shaped bacteria D) straight short rod shaped bacteria

The scanning electron micrographs supported the Gram staining results by showing that rod-shaped bacteria, identified as *C. thermopalmarium* by molecular identification in section 3.2.1.3, were predominant on the surface of the granule, but further illustrated the presence diplococcoid bacteria which were identified as Acidaminococcae bacterium DJF VR01 (Figure 20) by the analysis of the 16S rDNA genetic sequence. *C. thermopalmarium* has rod-shaped cells which are 0.7-1µm width and 2.0-8µm in length, this is depicted by the SEM image of the granules in Figure 16 with various sized rod-shaped bacteria (Soh *et al*., 1992). Acidaminococcae are usually diplococcoid, which are sometimes oval or kidney shaped with a cell diameter of 0.6-1µm (Rogosa, 1969).

3.2.1.3. Molecular Identification

PCR-DGGE was used to identify HPB present in the bioreactors. DNA extracted from the raw sample from the bioreactor, represented by lane PS, and from pure cultures, represented by lane PP, were analysed on a 20-70% gradient gel.

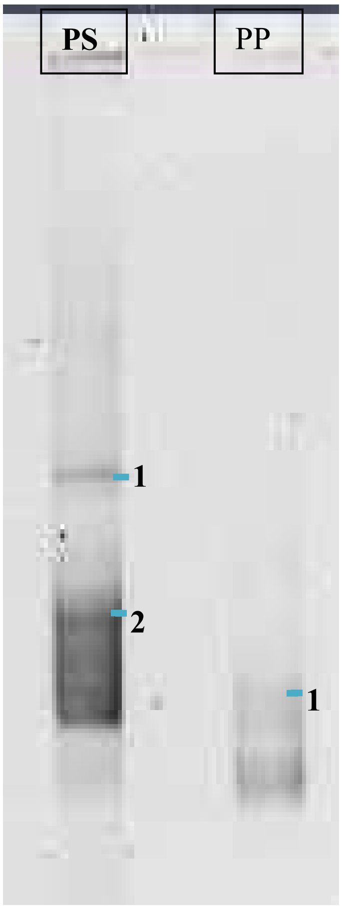


Figure 17: DGGE profile of the 16SrDNA fragment of the biohydrogen producing Anaerobic Fluidized Bed Bioreactor 1 with the internal bioreactor temperature at 55°C
Lane “PS” represents the raw sample DNA profiling while “PP” representing culturable bacteria.

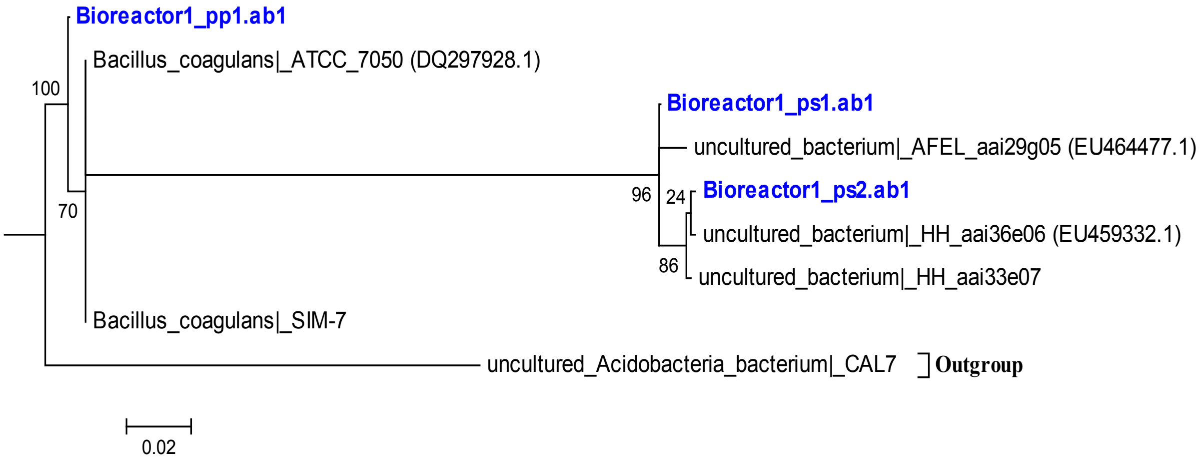


Figure 18: Phylogenetic tree derived from aligned 16S r DNA sequences of the excised DGGE bands (written in blue) of amplified 16S rDNA genes of bacterial consortium from Bioreactor 1 when the internal temperature of the bioreactor was 55°C. GenBank accession numbers are in brackets. The rooted tree is based on the Ribosomal Database project Jukes-Cantor distance model using Neighbor-joining algorithm with 100 bootstrapping. The scale bar represents 0.02 nucleotide divergence.

DNA analysis revealed that the culturable bacteria represented by band PP1 was closely affiliated with *Bacillus coagulans* ATCC 7050 strain with a sequence similarity of 98%. Band PS1 and PS2 (Figure 17) were closely affiliated with uncultured *Citrobacter* AFEL_aai29g05, with 100% similarity, and uncultured *Shigella* HH_aai36e06, respectively (Figure 18).

Molecular evaluation further revealed the presence of two unculturable bacteria in the original sample as uncultured *Citrobacter* AFEL_aai29g05 and uncultured *Shigella* HH_aai36e06, respectively. *Citrobacter* is a facultative anaerobe, which is capable of producing hydrogen using glucose as a substrate at pH 6.5-7 (Oh (b) *et al*., 2003). Huang *et al.* (1985) found that 58% of the Enterobacteriaceae in sewage sludge that are hydrogen producers were *Citrobacter* spp and 28% *Enterobacter* spp with only 0.3% being *Escherichia* spp (Huang *et al*., 1985).

Bacterial species such as *Klebsiella*, *Citrobacter*, *Shigella* and *E. coli* which are *Enterobacteria* were found growing in the AFBRs at thermophilic temperatures despite their classification as mesophiles (Droffner *et al*., 1995; Bonjoch and Blanch, 2009). Briton *et al.* (1994) observed that thermophillic compost where the starting material originates from human waste contained potential pathogenic species. These species were from the families Enterobacteraceae and Pseudomonadaceae, which include *E. coli, Serratia marcesens, Citrobacter freundii, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Briton *et al*., 1994; Droffner and Briton, 1994). Doffner *et al.* (1994) found that *E. coli* and *Salmonella* could survive at 60°C for 59 days during thermophilic compositing (Droffner and Briton, 1994). It was also found that these bacteria became undetectable when the temperature of the system was decrease from 62°C to 40°C (Droffner and Briton, 1994). The *E. coli* strain in that system survived atleast 9 days at 6-70°C in wastewater sludge compost (Droffner and Briton, 1994). Doffner *et al.* (1994) isolated an *E. coli* which was capable of growth at 65°C (Briton *et al*., 1994; Droffner and Briton, 1994). Doffner *et al.* (1995) found that *E. coli*, *Salmonella typhimurium* and *P. aeruginosa* create mutants that grow at 54°C (Briton *et al*., 1994; Droffner *et al*., 1995). These strains carried a genetic operon, called *cel*, which is responsible for the degradation of cellobiose and is only expressed at 48°C and above, suggesting a genetic basis for survival at elevated temperatures (Briton *et al*., 1994). Bonjoch and Blanch (2009) found that *E. coli* was the most frequent faecal coliform, followed by *Citrobacter* spp. in wastewater sludge composited at 55°C (Bonjoch and Blanch, 2009). Moreover, *Citrobacter* spp presented the highest resistance among identified faecal coliform following pasteurization at 60°C for 90 minutes and 80°C for 60 minutes, or compositing at 55°C (Bonjoch and Blanch, 2009).

The PCR amplified DNA obtained from the raw sample from Bioreactor 1 at 65°C was loaded in duplicate, lane 2 and 4, (Figure 19). Similarly the DNA isolated from the liquid culture was loaded in duplicate, lane 1 and 5, and the DNA isolated from a single colony on solid media also loaded in duplicate in lane 3 and 6 (Figure 19).

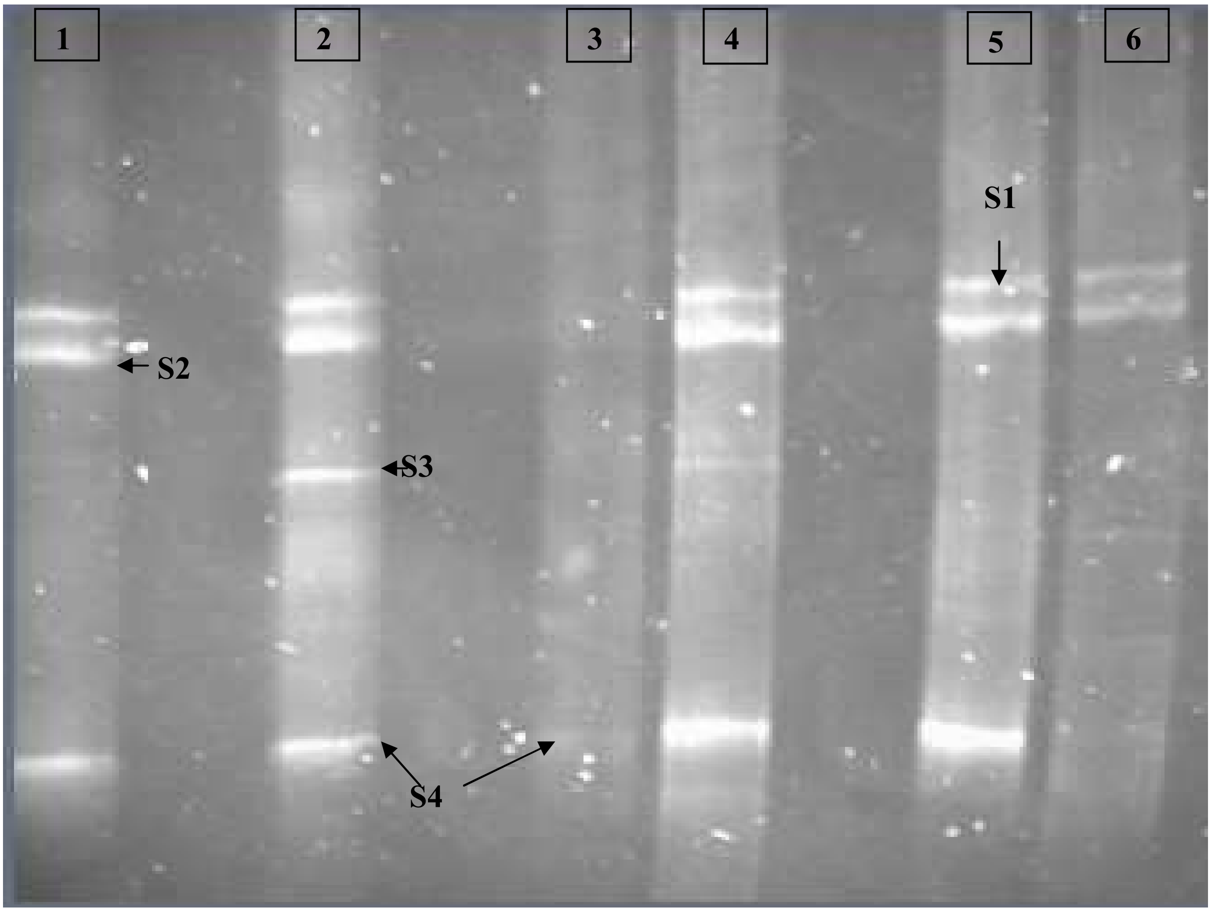


Figure 19: DGGE profile of 16S rDNA fragments of Bioreactor1 with the internal temperature of the reactor 65°C. Lane 1 and 5, DNA isolated from liquid media; lane 2 and 4, DNA isolated from the raw sample; Lane 3 and 6, DNA isolated from single colonies on solid media.

Bands S1, S2 and S3 (Figure 19) were closely affiliated with *Clostridium thermopalmarium* with a sequence similarity of 98% and to *Clostridium thermobutyricum* with a sequence similarity of 98%. However, since the sole carbon source of the medium was sucrose it was concluded that the bands represent *Clostridium thermopalmarium* strains because *Clostridium thermobutyricum* requires yeast for growth (Weigel *et al*., 1989) and cannot utilize sucrose (Soh *et al*.,1992). Furthermore *Clostridium thermobutyricum* stains Gram-positive while *Clostridium thermopalmarium* stains Gram- negative even at early stages of growth as depicted by Figures 14 and 15 (Wiegel *et al*., 1989; Soh *et al*., 1991). Band S4 was closely affiliated to *Acidaminococcaceae* bacterium DJF VR01 (Figure 20).

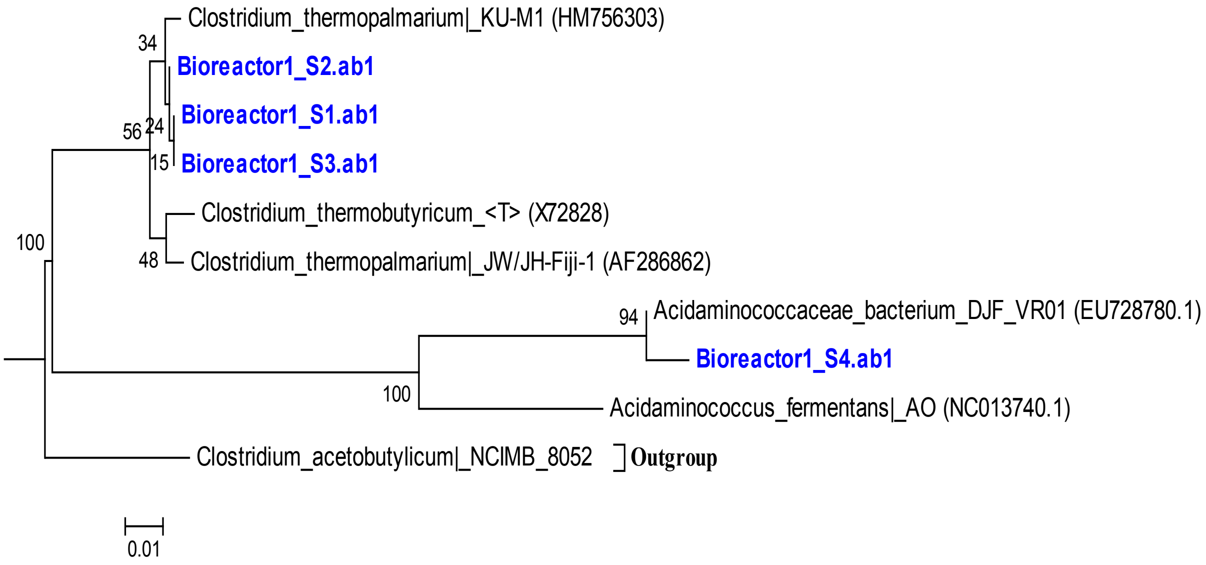


Figure 20: Phylogenetic tree derived from aligned 16S r DNA sequences of the excised DGGE bands (written in blue) of amplified 16S rDNA genes of bacterial consortium from Bioreactor 1 when the internal temperature of the bioreactor was 65°C. GenBank accession numbers are in brackets. The rooted tree is based on the Ribosomal Database project Jukes-Cantor distance model using Neighbor-joining algorithm with 100 bootstrapping. The scale bar represents 0.01 nucleotide divergence.

All the AFBRs had isolates (Figure 20, 28 and 36) affiliated with Acidaminococcaceae at the desired thermophilic temperature of 65°C. Rogosa stated that Acidaminococcaceae did not survive heating at 60°C for 30 minutes (Rogosa, 1969), however, even after pretreating the inoculum at 100°C for 45 minutes *Acidaminococcus* spp had been detected in all AFBRs. These bacteria ferment glutamate to ammonium, CO_2, acetate, butyrate and H_2 (Härtel and Buckel, 1996). Jumas- Bilak *et al*. (2007) discovered that *Acidaminococcus* spp can utilize citrate to produce hydrogen and hydrogen sulphide (Jumas- Bilak *et al*., 2007) It has a higher HPR than obligate anaerobes such as *Clostridium* species; however, it only produces 1mol H_2 per gram of glutamate whereas *Clostridium* species produce 4mol H_2/ g glucose (Chang *et al*., 2008). Figure 16 clearly illustrates the presence of oval diplococci, which is one of the morphological forms found in Acidaminococcaceae (Rogosa, 1969). As the inoculum had been pretreated by heat it is likely that Clostridial strains should be the major H_2 producers present in the system (Chang *et al*., 2008). However, *Clostridium* species were only isolated from Bioreactor 1. This might be due to short HRT values ranging from 2-4h for Bioreactor 2 and 3, whereas Bioreactor 1 HRT had values ranging from 1-10 and at the point of sampling the HRT was 6.5h. According to Chang *et al.* (2008) when bioreactor has an HRT of 6 then *Acidaminococcus* and *Clostridium* species are present at an equal ratio and the *Clostridium* species become the dominant H_2 producers at high HRTs (Chang *et al*., 2008). The *Clostridium thermopalmarium* affiliated bacteria were culturable, thus they can be used for bioreactor enrichment. *Clostridium thermopalmarium* was first identified by Soh *et.al* (1991) as a moderate thermophile with an optimum growth temperature of 50-55°C with an upper limit of 60°C, whereas Rainey *et al.*(1993) found the temperature optimum for it at 55°-57°C (Soh *et al*., 1992; Rainey *et al.,* 1993; Akao *et al*., 2007), however, it survived at 65°C within the bioreactor system. Rainey *et al.* (1993) found that *C. thermopalmarium* has a maximum growth temperature above 65°C (Rainey *et al.,* 1993). It can survive at pH ranges of between 6 and 8.2 (Akao *et al*., 2007). *C. thermopalmarium* forms terminal and subterminal elliptical endospores which slightly swell the cells (Soh *et al*., 1992; Rainey *et al.,* 1993), clearly depicted by Figure 14. *C. thermopalmarium* is known to produce butyrate directly from carbohydrates, with hydrogen and carbon dioxide as the major by products and small amounts of acetate, ethanol and lactate during the fermentation (Rainey *et al.,* 1993; Akao *et al*., 2007). The microbial shift for Bioreactor1 was from facultative anaerobes at the initial phase of the operational period at 55°C, to obligate anaerobes *Clostridium thermopalmarium* and Acidaminococcaceae. Thus initial hydrogen production was from *Bacillus coagulans* and *Citrobacter*, when the gas was analysed 38.6% of the total gas comprised of hydrogen. Hydrogen concentration was 55% of the total gas when *Clostridium thermopalmarium* and Acidaminococcaceae were the hydrogen producers at 65°C.

3.2.2 Bioreactor 2

3.2.2.1 Gram Staining

Gram staining of culturable bacteria revealed singular and binary rod- shaped bacteria. Both Gram- positive bacteria, labeled A, and Gram- negative, labeled B, were observed.

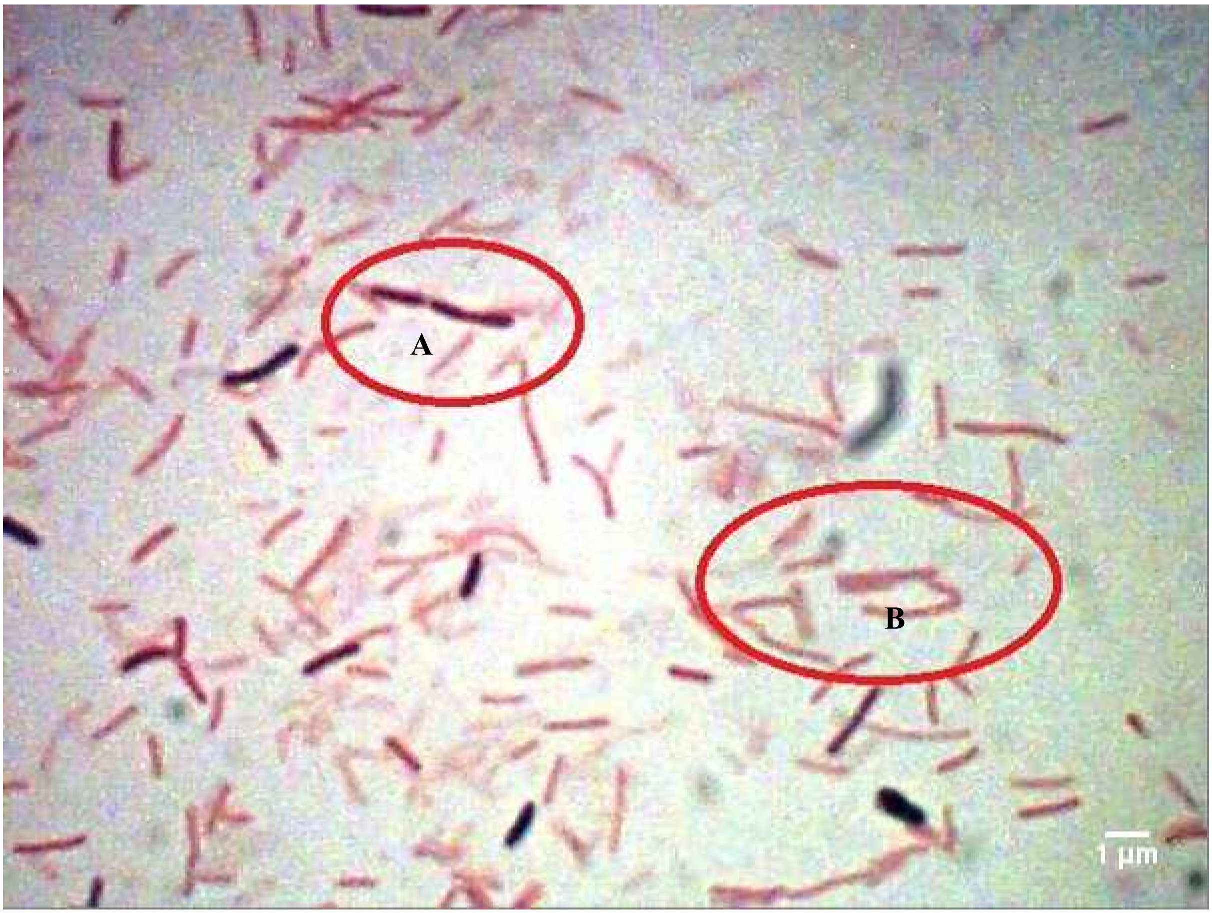


Figure 21: Gram stain of Bioreactor 2 culturable bacteria with the internal temperature of the bioreactor at 55°C

Molecular identification (Figure 26) identified the culturable bacteria as *Bacillus subtilis* MJ4 and *Klebsiella* spp. TPS10. Bacillus subtilis is a Gram- positive bacterium represented by "A" in Figure 21, whereas Klebsiella spp. are Gram- negative bacterium represented by "B" in Figure 21.

Gram staining of culturable bacteria when the operational temperature was 65°C revealed Gram- negative rod shaped bacteria occurring in binary rods and chains.

Figure 22: Gram stain of Bioreactor2 culturable bacteria colony 1 with the internal temperature of the bioreactor at 65°

Two types of colonies were observed on the solid media, nutrient agar (Biolab®, Merck (Pty)Ltd, Wadeville, Johannesburg, South Africa), these were identified as colony 1 and colony 2 were identified to be *Bacillus coagulans* SL5 for colony 1 and *Bacillus coagulans* SIM7 for colony 2 with 100% sequence similarity. *B. coagulans* is a Gram- positive bacteria, however, the cells stained Gram- negative for both the colony smear (Figure 22) and the liquid culture (Figure 23), which might be as a result of overstaining.

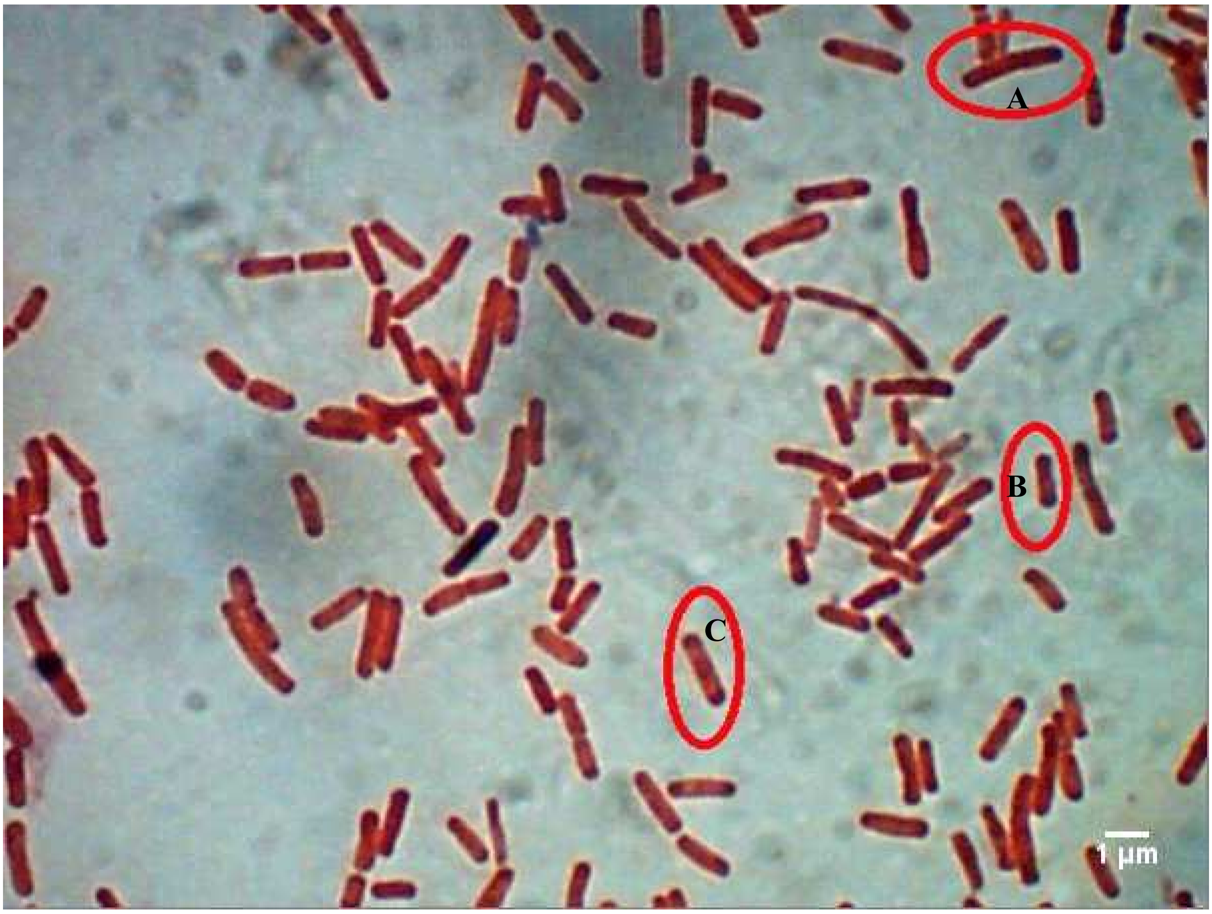


Figure 23: Gram stain of Bioreactor 2 culturable bacteria in liquid medium with the internal temperature of the bioreactor at 65°C

The liquid culture cells (Figure 23) appear to be shorter than the colony cells (Figure 22). The colony cells (Figure 22) for *B. coagulans* appeared as a large network of chains whereas the cells in the liquid culture (Figure 23) appear is binary cells and single cells with varying cell sizes, depicted by A, B and C (Figure 23).

3.2.2.2. Scanning Electron Microscopy

Rod shaped bacteria were predominant on the surface of the hydrogen producing granules, cells C, D and E, diplococcoid bacteria, cell A, and coccoid chains, cell B, were also present on the granule surface as well.

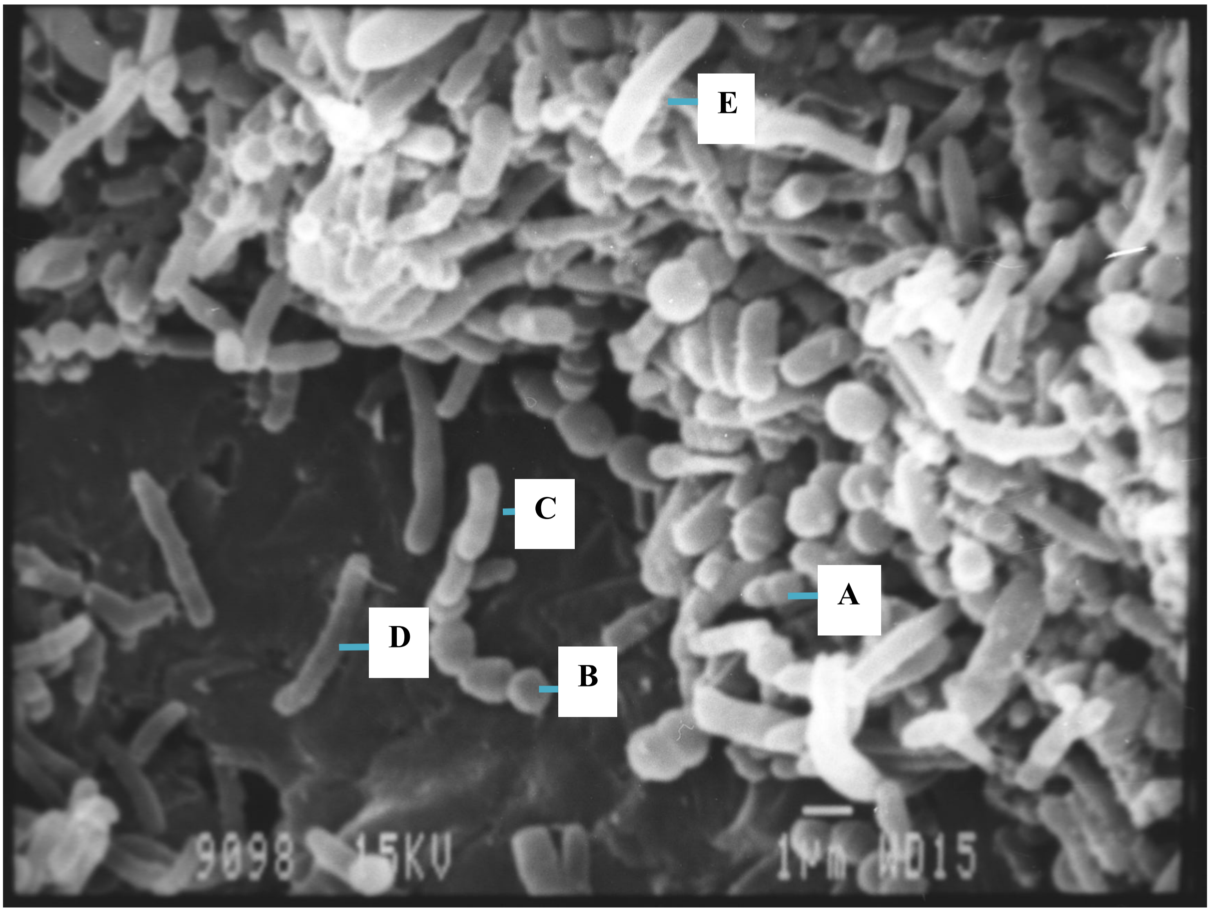


Figure 24: SEM micrograph of Bioreactor 2 granule depicting A) diplococcoid bacteria B) coccoid bacteria C) short rod shaped bacteria D) thin long rod shaped bacteria E) thick long rod shaped bacteria

Molecular analysis (Figure 28) identified four bacteria which are rod- shaped namely *E. coli*, *B. coagulans*, *Actinomyces* and *Bifidobacterium*. The coccoid chains were identified as *Selemonas ruminantium* in the morphological state that signifies that the bacteria have passed the stationary phase (Reinhard *et al*., 2005).

3.2.2.3 Molecular Identification

In PCR- DGGE every DNA band represents a different organism. DNA extracted from the raw sample from the bioreactor corresponds to lane LS while the DNA sample from pure cultures corresponds to lane LP were analysed on a 20-70% gradient gel.

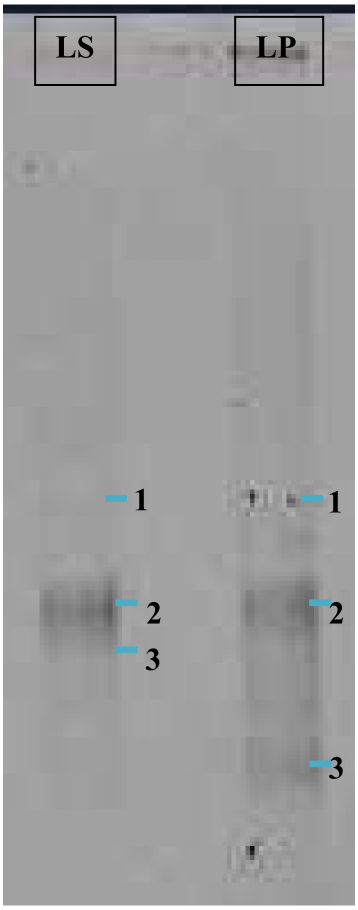


Figure 25: DGGE profile of the 16SrDNA fragment of the biohydrogen producing Anaerobic Fluidized Bed Bioreactor 2 with the internal bioreactor temperature at 55°C
Lane "LS" represents the raw sample DNA profiling while "LP" representing culturable bacteria

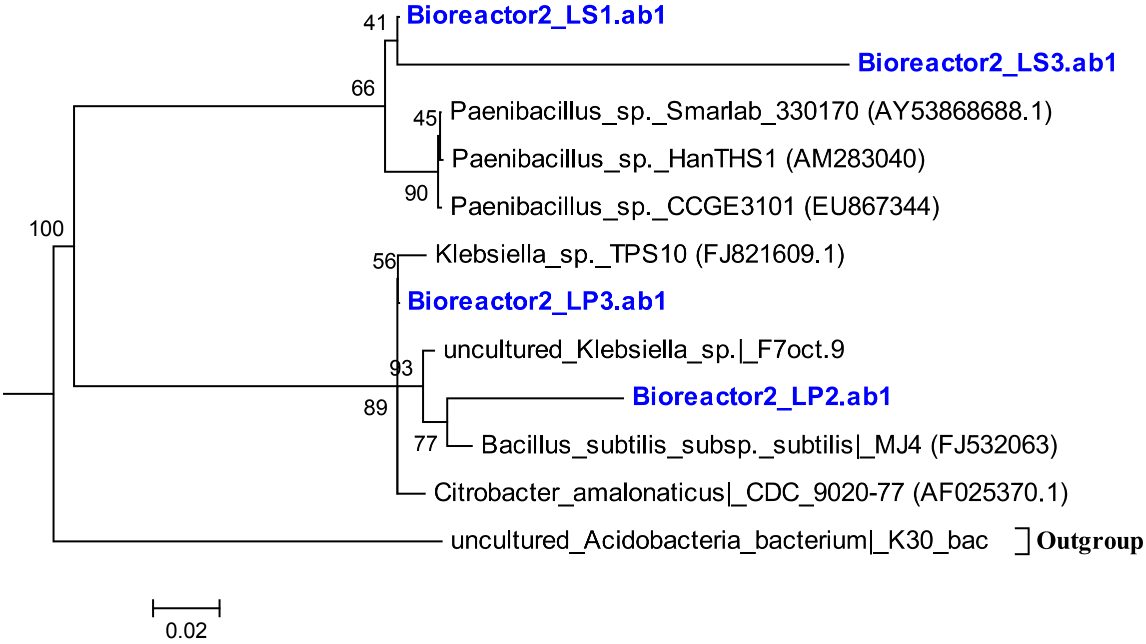


Figure 26: Phylogenetic tree derived from aligned 16S r DNA sequences of the excised DGGE bands (written in blue) of amplified 16S rDNA genes of bacterial consortium from Bioreactor 2 when the internal temperature of the bioreactor was 55°C. GenBank accession numbers are in brackets. The rooted tree is based on the Ribosomal Database project Jukes-Cantor distance model using Neighbor-joining algorithm with 100 bootstrapping. The scale bar represents 0.02 nucleotide divergence.

The raw sample separated into three bands as illustrated by lane LS, only two the bands, LS1 and LS3, were identified as *Paenibacillus* by molecular analysis. *Paenibacillus* is a nitrogen-fixing facultative anaerobe that utilizes sucrose (Rheims *et al.*, 1991). It is capable of forming endospores under anaerobic conditions (Rheims *et al.*, 1991).

Band LS1 and LS3 (Figure 25) were strains of the same species with a bootstrap value of 100% and closely affiliated with *Paenibacillus* sp. Band LP2 (Figure 25) was closely affiliated with *Bacillus subtilis* MJ4, whereas Band LP3 (Figure 25) was closely affiliated with *Klebsiella* spp. TPS10 with a sequence similarity of 100%.

Orlygsson *et al.* (2010) found that *Paenibacillus* sp. which has a reported optimum growth temperature of 28- 30°C were present in the ethanol- producing enrichment cultures which were grown at 50°C and 60°C for a week (Orlygsson *et al.*, 2010). Some *Paenibacillus* sp. have also proven to be cellulolytic and are capable of producing ethanol from sugar fermentation, producing approximately 1 mol ethanol/ g glucose (Orlygsson *et al.*, 2010).

Droffner *et al.* (1995) found two *Klebsiella pneumoniae* strains when sampling compositing cow manure and in yard waste compost (Droffner *et al.*, 1995). When sampling compositing cow manure on day four of compositing at temperatures over 60°C *Klebsiella pneumoniae ss ozaenae* was isolated while *Klebsiella pneumoniae ss pneumoniae* was isolated on day 29 of compositing yard waste at 59°C (Droffner et al., 1995). Mason and Hamer (1987) found that *Klebsiella pneumoniae* was capable of cryptic growth when exposed to thermophilic temperatures of 55°C and 60°C, where bacterial growth resumed when new media was added to the bioreactor (Mason and Hamer, 1987). Band LP2 was affiliated with Bacillus subtilis MJ4 strain, a thermophilic *Bacillus* species (Shukla and Kar, 2006). Thermotolerant Bacillus have a optimum growth temperature lying between 45-70°C. These produce important enzymes such as amylases, cellulases and xylanases, which degrade most abundant natural polymers (Shukla and Kar, 2006; Pakpitcharoen *et al.*, 2008). Therefore *Bacillus subtilis* can serve as a primary fermenter in the bioreactor. Shukla *et al.* (2006) found that *B. subtilis* had stable amylolytic enzyme activity at 60°C when degrading potato peels with a pH range of between 5-7 (Shukla and Kar, 2006). The degradation of starch would be important when food production effluents will be used as a nutrient source for hydrogen production. Pakpitcharoen *et al.* (2008) found that *B. subtilis* had an optimal growth temperature of between 45-70°C, with a high xylanase activity and high cellulose hydrolization activity (Pakpitcharoen *et al.*, 2008). Tiwari et al (2012) found that *B. subtilis* was thermotolerant,at temperatures between 40-50°C, when decolourizing molasses (Tiwari *et al.*, 2012). The *B. subtilis* strain was capable of sucrose degradation, since molasses is 40% sucrose, and melanoidin degradation (Tiwari *et al*., 2012). Melanoidin, a natural browning polymer, is responsible for the dark brown colour of molasses (Tiwari *et al*., 2012).

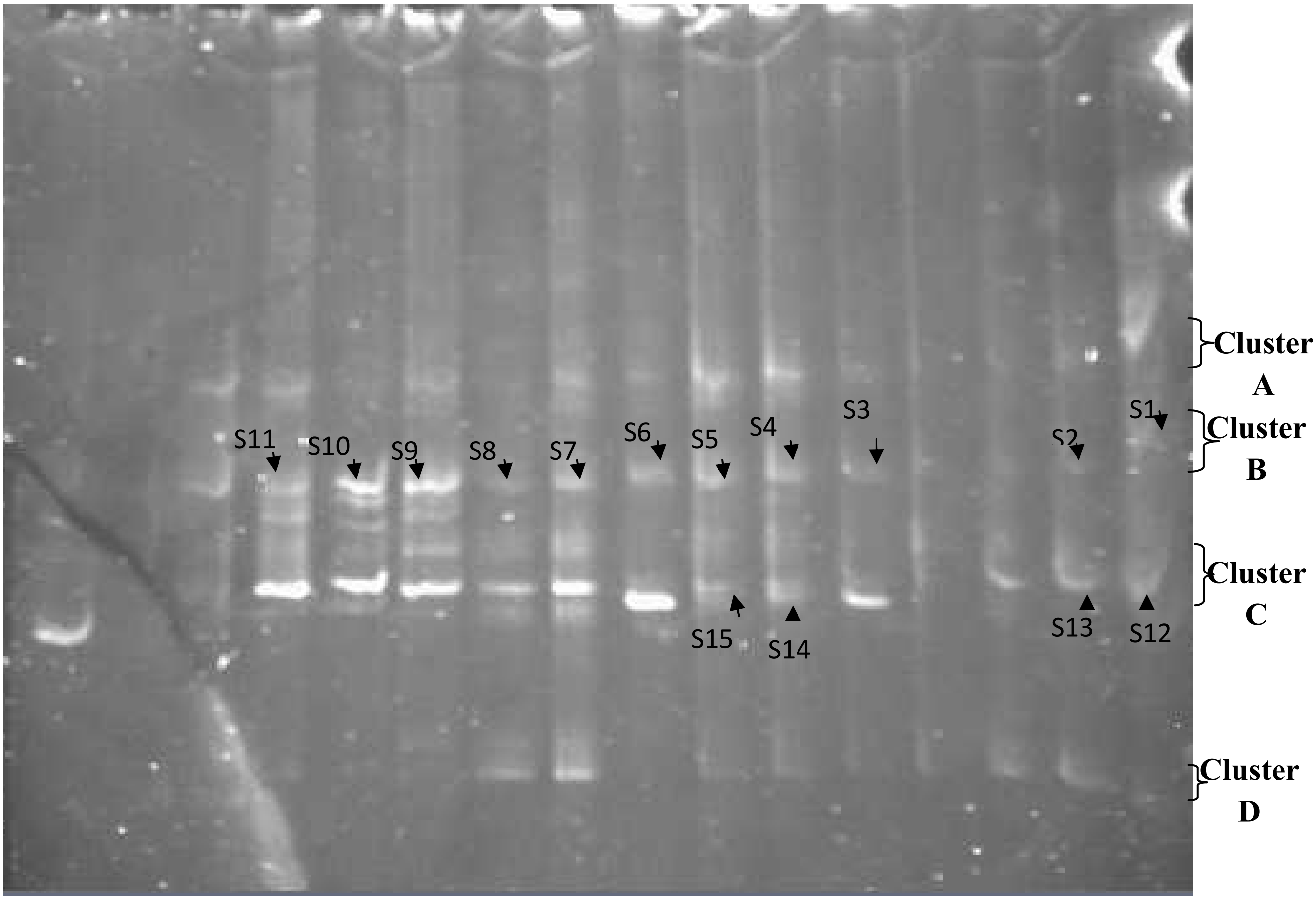


Figure 27: DGGE profile of 16S rDNA fragments of Bioreactor 2 with the internal temperature of the reactor 65°

Four predominant bands, illustrated by clusters A, B, C and D in Figure 27, were observed of the DGGE DNA profiling of the microbial community in the bioreactor at 65°C. However, only Cluster B and Cluster C were visible on the UV transilluminator when bands were being excised from the gel. The full range of bands was excised and DNA was eluted overnight at 4°C. The DNA was reamplified and sent for DNA sequencing, however, only the labelled bands (Figure 27) were successfully identified.

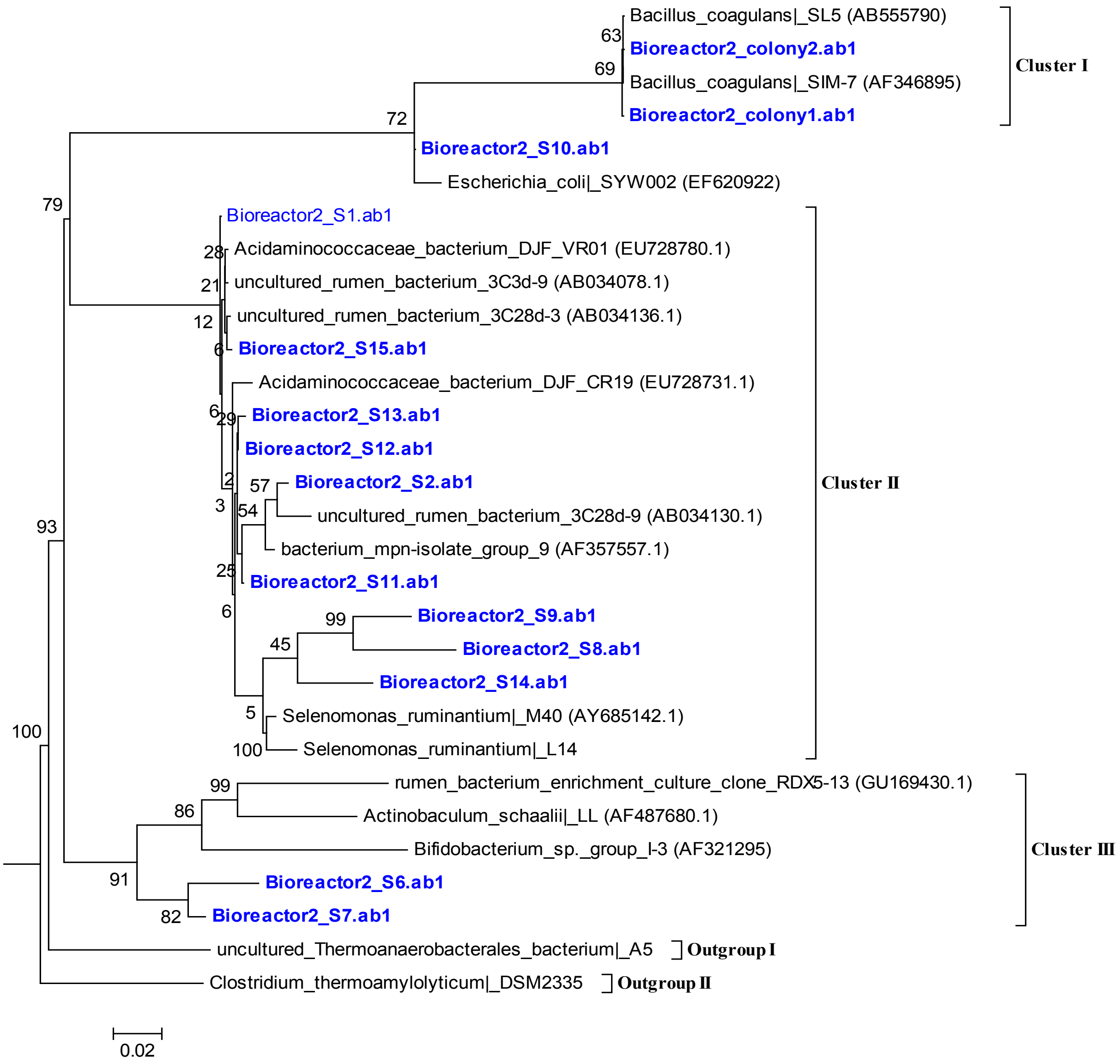


Figure 28: Phylogenetic tree derived from aligned 16S r DNA sequences of the excised DGGE bands (written in blue) of amplified 16S rDNA genes of bacterial consortium from Bioreactor 2 when the internal temperature of the bioreactor was 65°C. The tree is based on the Ribosomal Database project Jukes-Cantor distance model using Neighbor-joining algorithm with 100 bootstrapping. The scale bar represents 0.02 nucleotide divergence. Two outgroups were selected, uncultured *Thermoanarobacterales* and *Clostridium thermoamylolyticum*

DGGE profiling of biohydrogen- producing granules revealed the presence of bacteria that fall in 3 phyla Firmicutes, Proteobacteria and Actinobacteria. In the phylum Firmicutes 2 classes of bacterial clusters were identified as part of the biohydrogen- producing consortium as *Bacillus* and *Clostridia*, in the phylum Proteobacteria, *Gammaproteobacteria* and in *Actinobacteria* phylum, *Actinobaculum.* and *Bifidobacteria.* Three clusters (Figure 28) were observed, Cluster I represents the culturable bacteria with were identified as *Bacillus* *coagulans* SIM 7 SL5 for colony 1 and *Bacillus coagulans* SL5 for colony 2. Cluster II consisted of bacteria that fell under the family of Veillonellaceae, which are Gram- negative lactate fermenting, anaerobic coccid bacteria, namely *Selemonas ruminantium* and *Acidaminococcus* spp . Cluster III comprised of rumen bacteria of the family Actinobacteria, namely *Actinobaculum schaalii* and *Bifidobacterium* I3 strain.

Band S1 (Figure 28) was closely affiliated with *Acidaminococcaceae* bacterium DFJ VR01. Band S2 was affiliated with uncultured bacterium 3C28d-9, this bacterium had 99% sequence similarity with *Selemonas bovis* and *Acidaminococcaceae* bacterium DFJ VR01. Band S6 and S7 (Figure 28) are strains f the same bacteria and were closely affiliated with *Bifidobacterium* sp I3. This *Bifidobacterium* strain had a 100% similarity to *Bifidobacterium thermacidophilum* (AB437362.1). Band S8, S9 and S14 were strains of each other and these were closely affiliated with *Selemonas ruminantium* M40. These were also closely linked to band S11, which was affiliated with bacterium mpn isolate group 9. This bacterium had 98% sequence *Selemonas rumanintium*. Band S10 as closely affiliated to E. coli SYW 002, however this was ruled out as contamination as it doesn't fall within the three major clusters evident on the sample range as seen in Figure 28. Band S12 and S13 were strains of each other and were closely affiliated to *Acidaminococcaceae* bacterium DFJ CR19. Band S15 was closely affiliated with uncultured bacterium 3C28d-3, which had a 98% sequence similarity to *Acidaminococcaceae* bacterium DFJ CR19 and 99% similarity *Selemonas bovis*.

B. coagulans was the culturable bacteria, which is H_2- producing bacterium as discussed previously in Section 3.1.2.1 (Kotay and Das, 2007). Six isolates in bioreactor 2 were closely affiliated with *Selemonas* spp., four afflitiated with *Selemonas ruminantium* and two with *Selemonas bovis*. *Selemonas ruminantium* is a secondary fermenter that converts intermediate substrates such as succinate and lactate to propionate, acetate, CO_2 and for some strains trace amounts of H_2 (Scheifinger *et al*., 1975; Russell and Rychlik, 2001; Zhang and Dong., 2009). *S. ruminantium* is a pH- resistant ruminal bacterium. It achieves this resistance by allowing its intracellular pH to decline which protects it from the influx and accumulation of fermentative acidanions (Russell and Rychlik, 2001). Hydrogen formation is substantially increased in strains that produce trace amounts of H_2 where co-culturing with hydrogen consuming bacteria such as methanogenic bacteria (Scheifinger *et al*., 1975). *S. ruminantium* are spiral in shape, however, another morphological variant has been observed in cultures that have passed the stationary phase (Kingsley and Hoeniger, 1973). These variant forms are round, which may occur in singles or in chains as indicated by Figure 23 (b) (Kingsley and

Hoeniger, 1973). However since no free H_2 accumulates, the quantity of H_2 formed can be calculated from the amount of methane produced (Scheifinger *et al.*, 1975). Zhou *et al.* (2012) found that *Selemonas ruminantium* M40 used hydrogen as an electron donor for an elevated capacity for fumarate reduction (Zhou *et al.*, 2012). *S. ruminantium* M40 degrades to produce acetate and proprionate (Zhou *et al.*, 2012). Ravindran *et al.* (2010) found that *S. ruminantium* HM00123 hydrolysed ANFL, animal fleshing proteinaceous solid waste generated in the manufacturing of leather, in 7 days at pH6.6 (Ravindran and Sekaran., 2010) . This strain had 94% sequence similarity with *Acidaminococcaceae* bacterium DFJ VR01. Ravindran *et al.* (2010) identified *S. ruminantium* HM00123 as an obligate anaerobic, Gram-negative, non- spore forming, rod- shaped bacterium capable of surviving at 45°C and could hydrolise protenacious substrates (Ravindran and Sekaran., 2010). Selemonas bovis is a Gram- negative, strictly anaerobic, non- spore forming, crescent- shaped bacterium (Zhang and Dong, 2009). Zhang *et al.* (2009) found that S. bovis was capable of cellulose degradation (Zhang and Dong, 2009). It also ferments, glucose, sucrose and lactose (Zhang and Dong, 2009. Zhang *et al.* (2009) found that it degrades glucose to acetate, propionate and CO_2 at a ratio of 0.7:1.2:1.2 for actetate:propionate: CO_2 per mole of glucose (Zhang and Dong, 2009). Band S6 and S7 (Figure 28) were affiliated with *Bifidobacterium* group I3, which has a 98% sequence similarity to *Bifidobacterium thermacidophilum* and a bootstrap value of 86 with *Actinobaculum schaalii* (Figure 28). *Bifidobacterium thermacidophilum* is a rumen bacterium found in mammals and in anoxic environments such as sewage (Dong *et al.*, 2000). *B. thermacidophilum* is a Gram- positive, non- spore forming, thermotolerant obligate anaerobic bacterium (Dong *et al.*, 2000; Zhu *et al.*, 2003). It is a moderate thermophile capable at growing at 49.5°C at a pH as slow 4 (Dong *et al.*, 2000). *B. thermacidophilum* produces acetic acid and lactic acid from anaerobic glucose fementation, producing ratios of 2.46-2.72:1 acetic acid:lactic acid per mol of glucose (Dong *et al.*, 2000). It is also capable of fermenting sucrose and starch (Dong *et al.*, 2000). *B. thermacidophilum* has pleomorphic, rod-, club- and V-shaped cells with sizes that are 0.5µm in diameter and between 1.5-2.5µm in length (Dong *et al.*, 2000). *Actinobaculum schaalii* belongs to the genera *Actinomyces* (Reinhard *et al.*, 2005). *Actinomyces* are a heterogenous group of Gram- positive, non-spore forming rod-shaped bacteria which may appear filamentous, diphtheroidal or branching (Collins *et al.*, 2000; Nikolaitchouk *et al.*, 2000; An *et al.*, 2006; Ziganshin *et al.*, 2010). These bacteria are either facultative or obligate anaerobes that ferment carbohydrates such as glucose, fructose, lactose and sucrose (Collins *et al.*, 2000; Reinhard *et al.*, 2005;An *et al.*, 2006; Ziganshin *et al*., 2010). Strains such as *Actinomyces rumicola*, first isolated from cattle rumen, ferment carbohydrates forming formic acid as the main end product. Formate can be used as a substrate for hydrogen production. Acetate and lactate are also produced (An *et al*., 2006). *A. rumicola* is a nitrogen reducing bacterium that produces a number of enzymes that degrade organic material, lignin and chitin (An *et al*., 2006). *Actinobaculum schaalii* is a coccoid rod-shaped, Gram- positive, facultative anaerobic bacterium usually found in urinary infection speciments (Reinhard *et al*., 2005; Tschudin-Sutter *et al*., 2011). It can degrade glucose, maltose and sucrose (Reinhard *et al*., 2005). Only one isolate was affiliated to *E. coli*. The presence of facultative anaerobes such as *E. coli*, which consume trace amounts of oxygen, promotes the growth of obligate anaerobes (Koskinen *et al*., 2007). However, *E. coli* is a potential H_2 producer through mixed acid fermentation where it ferments glucose to CO_2, lactate, ethanol, acetate and H_2 (Koskinen *et al*., 2007).

3.2.3 Bioreactor 3

3.2.3.1. Gram staining

Gram staining of culturable bacteria revealed rod shaped singular and binary, Gram- negative bacteria.

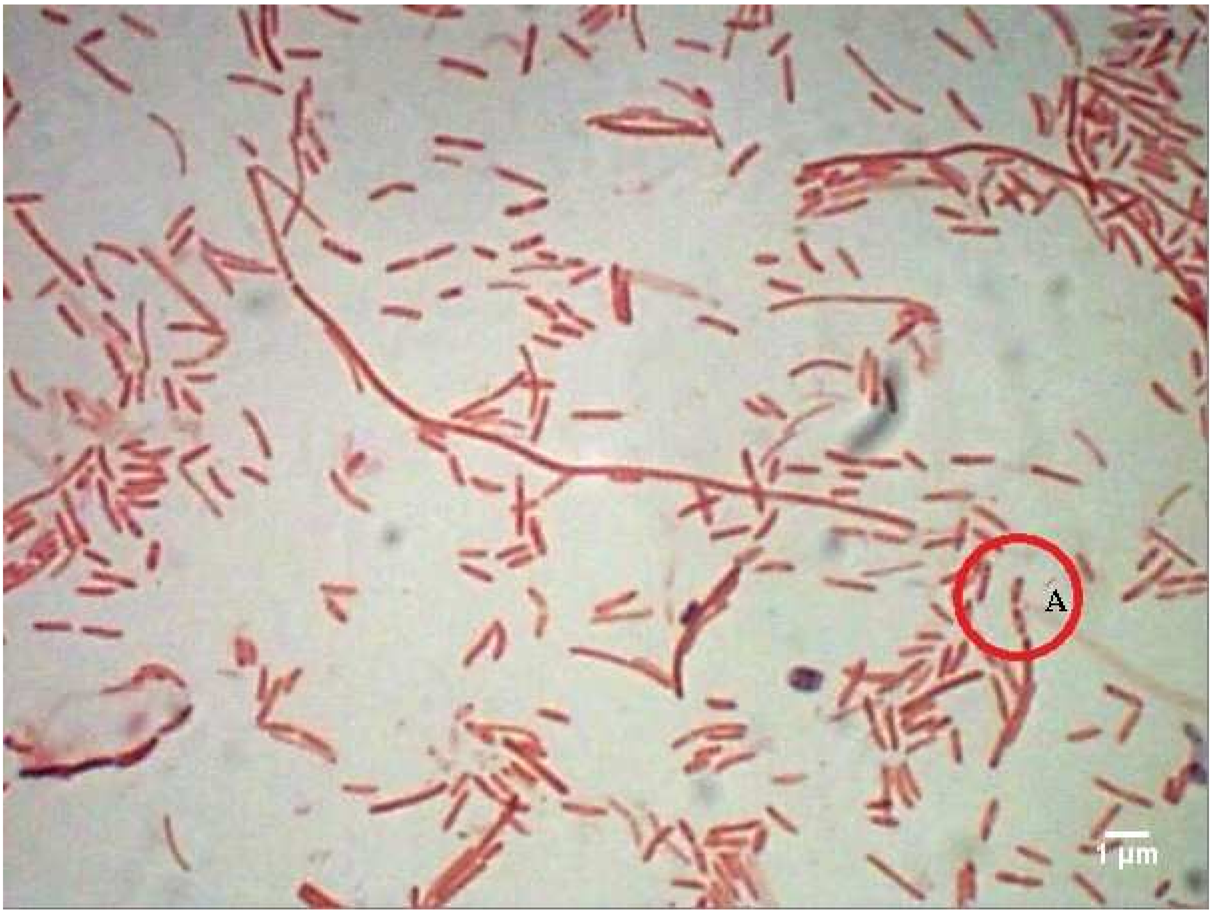


Figure 29: Gram stain of Bioreactor 3 culturable bacteria with the internal temperature of the bioreactor at 55°C

Molecular analysis identified the bacterium to be affiliated to an uncultured Rhodobacterium F3C99. *Rhodobacter* are asporogenic, Gram- negative, singular or binary rods that are mesophilic and facultative anaerobes (Zubova *et al*., 2007). However the Gram staining image shows bacterial cells with subterminal endospores, in cells labelled "A" (Figure 29). Only band FP3 (Figure 33) could be successfully identified (Figure 34), however 6 DNA bands are visible in lane FP in Figure 34. As the temperature was increased to 65°C four bands were successfully indentified (Figure 36), leading to the assumption that those species were present at 55°C but in low numbers which rendered them undetectable. *Bacillus* sp KHg2 appeared to be the only endospore forming bacteria identified in the system.

Culturable bacteria when the operational temperature was at 65° C were mostly Gram-positive, rod shaped bacteria (Figure 30) and bacteria that formed long filamentous chains (Figure 31). Some rod- shaped bacteria showed the presence of sub-terminal spores (Figure 25).

Figure 30: Gram stain of Bioreactor 3 culturable bacteria colony1 with the internal temperature of the bioreactor at 65°C

Molecular identification of the pure colony cultures (Figure 30) and on the liquid culture obtained from the pure colonies (Figure 31) identified the two samples as *Bacillus thermoamylovorans* (Figure 36). *B. thermoamylovorans* is a rod-shaped Gram- positive bacterium as illustrated by Figure 30 and 31, with cells that occur singly or in short chains. However Figure 30 also illustrates endospore forming bacteria, shown by cells labelled ‘‘A”, but *B. thermoamylovorans* is a non- spore forming bacterium (Combet-Blanc *et al*., 1995). However, molecular identification indicated the presence of a terminal endospore forming bacterium known as *Bacillus* sp KHg2.

Figure 31: Gram stain of Bioreactor 3 culturable bacteria in liquid media with the internal temperature of the bioreactor at 65°C

The liquid culture in Figure 31 only shows the presence of Gram- positive binary rods and rods that formed long filamentous chains. None the endospore forming bacteria was observed in the liquid culture. Molecular identification showed that the bacteria are *B. thermoamylovorans* a lactate producing bacteria (Combet-Blanc *et al*., 1995).

3.2.3.2. Scanning Electron Microscopy

The scanning electron micrographs of Bioreactor 3 granules showed only rod- shaped bacteria were present on the surface of the granules.

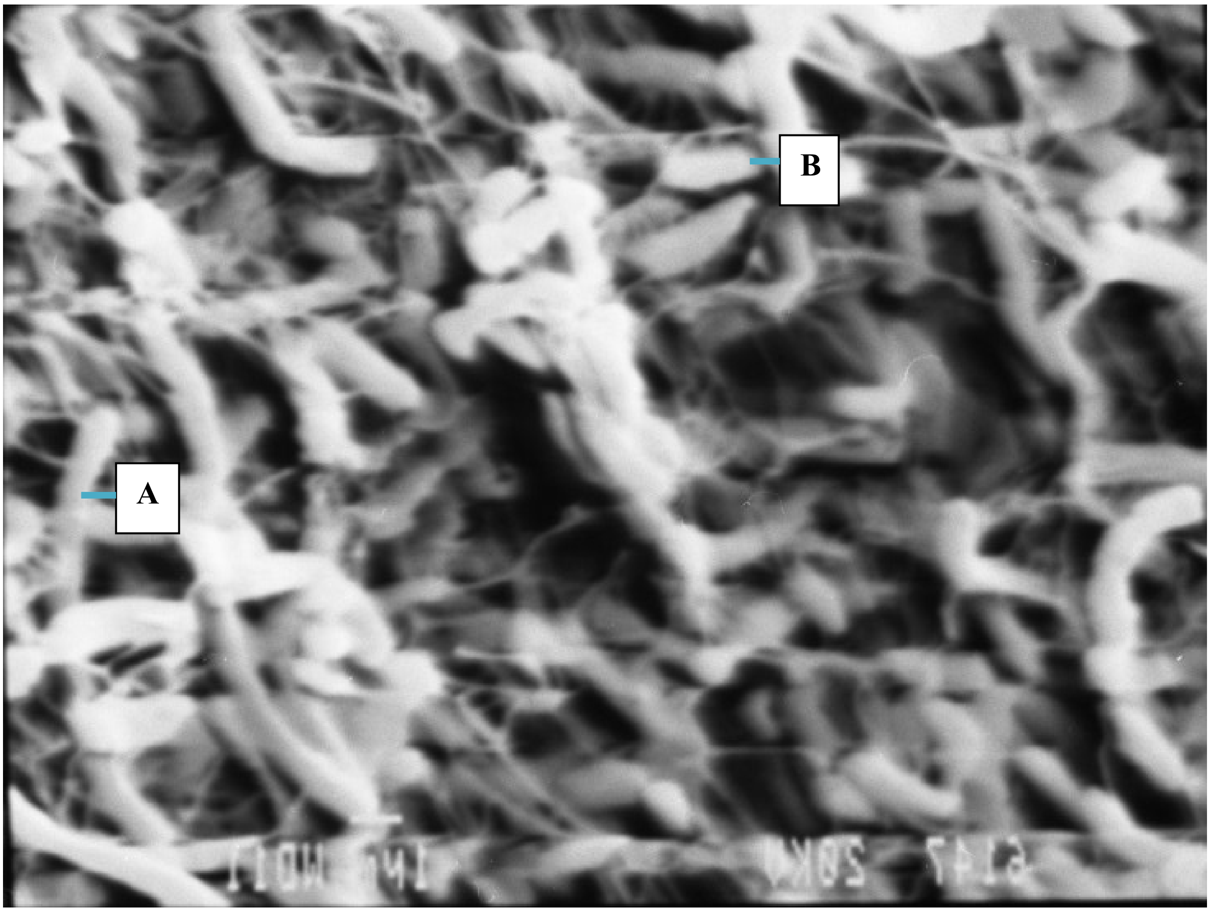


Figure 32: SEM micrograph of Bioreactor 3 granule depicting a) long rod shaped bacteria b) short rod shaped bacteria.

The SEM micrograph indicates the presence of long rod- shaped bacteria, however since the dominant bacteria was *B. thermoamylovorans*, we can assume that what appears to be a long bacteria could possibly be short chains (Combet-Blanc *et al*., 1995).

3.2.3.3. Molecular Identification

The raw sample in lane FS separated to give one predominant band and the culturable bacteria sample in lane FP separated into 3 bands, where FP3 was the only band that could be identified by molecular identification. Band FP3 is at the same position as FS1, which might mean they represent the same bacterium. Band FP3 was identified as an uncultured *Rhodobacterium* F3C99 strain (Figure 34).

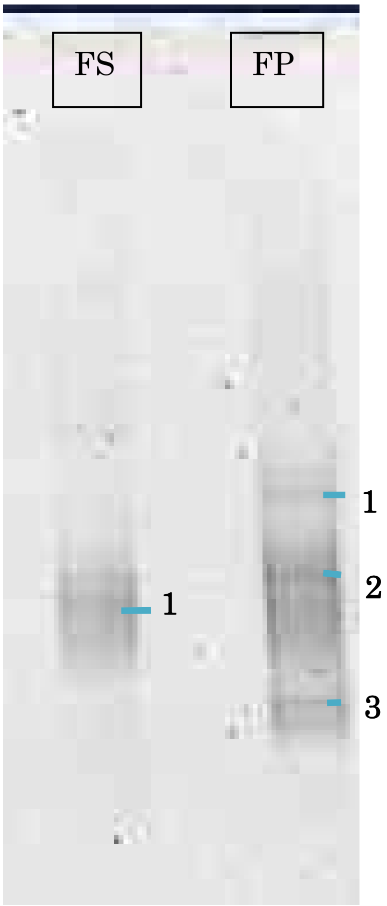


Figure 33: DGGE profile of the 16SrDNA fragment of the biohydrogen producing Anaerobic Fluidized Bed Bioreactor 3 with the internal bioreactor temperature at 55°C
Lane "FS" represents the raw sample DNA profiling while "FP" representing culturable bacteria

Only DNA eluted from band FP3 was able to be identified by molecular identification (Figure 33). Band FS1, FP1 and FP2 could not be identified as the DNA could not be eluted and reamplified.

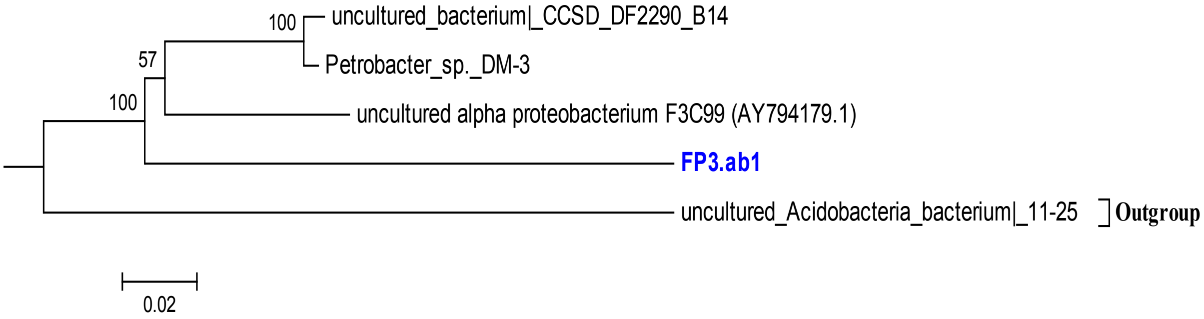


Figure 34: Phylogenetic tree derived from aligned 16S r DNA sequences of the excised DGGE bands (written in blue) of amplified 16S rDNA genes of bacterial consortium from Bioreactor 3 when the internal temperature of the bioreactor was 55°C. The tree is based on the Ribosomal Database project Jukes-Cantor distance model using Neighbor-joining algorithm with 100 bootstrapping. The scale bar represents 0.02 nucleotide divergence

The genus *Rhodobacter* has cell wall organization typical of Gram- negative bacteria (Zubova *et al*., 2007). Wei et al. (2010) found that *Rhodobacteria* are capable of producing 3.3 mol H_2/mol glucose (Wei *et al*., 2010). These bacteria multiply by binary division and can be present a singular cells, binary cells and chains (Zubova *et al*., 2007). These photosynthetic purple non- sulphur (PNS) bacteria evolve molecular hydrogen catalysed by nitrogenase under nitrogen deficient conditions using light energy and reduced compounds (organic acids) such as malate and lactate (Fedorov *et al*., 1998; Koku *et al*., 2002; Levin *et al*., 2004). Yi□it *et al*. (1999) found that *Rhodobacterium sphaeroides* O.U. 011 is able to produce hydrogen anaerobically upon illumination (Yi□it *et al*., 1999). Tsygankov *et al*. (1994) found that an immobilized continous culture of *Rhodobacter sphaeroides* 1.3 L H_2/ml/ h (Tsygankov *et al*., 1994; Levine *et al*., 2004). Even though PNS bacteria prefer a photoheterotrophic growth mode, they are capable of several alternative metabolic modes such as aerobic/ anaerobic respiration, photoautotrophy and fermentation (Koku *et al*., 2002). Normally in a photobioreactor the conditions are adjusted to favour photoheterotrophic mode of growth, however if the light availability is poor or the experiment is carried out under natural light, then the bacteria may switch to a fermentative mode of metabolism (Koku *et al*., 2002). Furthermore it has been observed evidence of substrate utilization in the dark periods possibly indicating fermentative metabolism (Koku *et al*., 2002). A study by Tao *et al*. (2007) employed photofermentation with *Rhodobacterium sphaeroides* SH2C to convert the fatty acids produced during dark fermentation into hydrogen and increased the total H_2 yield from 3.7mol H_2/mol sucrose in dark fermentation to 6.6 mol H_2/mol sucrose by using a two step dark fermentation process (Tao *et al*., 2007; Uyar *et al*., 2009). Bacteria of the superfamily Rhodospirillaceae are capable of growing in the dark using CO as the sole carbon source to produce ATP with the release of hydrogen and CO_2 (Levin *et al*., 2004). Hydrogen production rates by photoheterotrophic bacteria increase in systems where the bacteria are immobilized as seen in a system where Rhodopseudomonas capsulate and *Rhodobacter sphaeroides* were co-cultured on activated glass produced 100% more hydrogen per hour (Levin *et al*., 2004). The hydrogen production increased from 50ml H_2/ml/ h to 100 ml H_2/ml/ h (Levin *et al*., 2004).

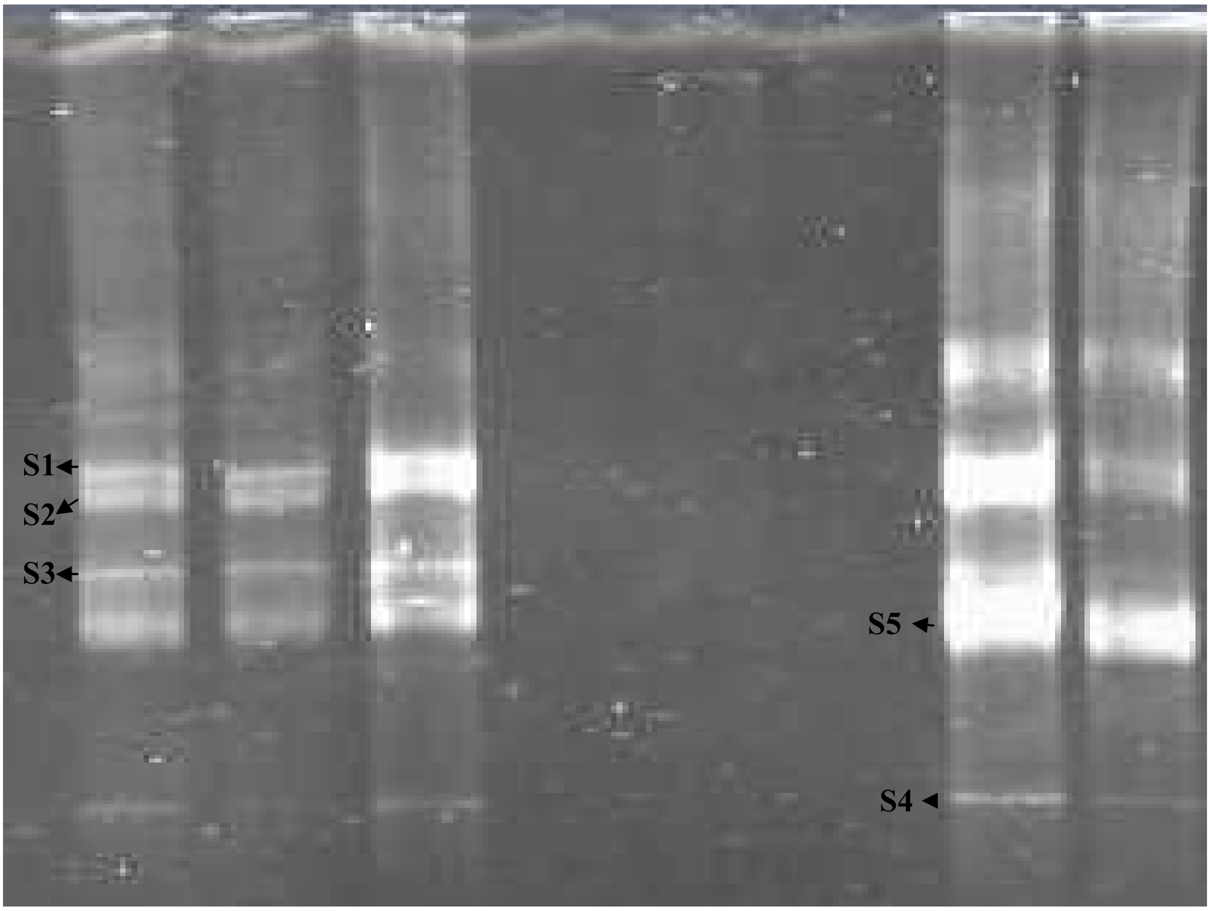


Figure 35: DGGE profile of 16S rDNA fragments of Bioreactor3 with the internal temperature of the reactor 65°C

Band S1 and S2 (Figure 35) were associated with culturable bacteria and molecular identification showed that the bands were affiliated with *Bacillus thermoamylovorans* (Figure 36). Band S3 was affiliated with *Acidaminococcaceae* bacterium DFJ CR19. Band S4 was affiliated with Bacillus sp. KHg2 and Band S5 was affiliated with an uncultured unclassified *Rhodocyclaceae* strain UWNR3.

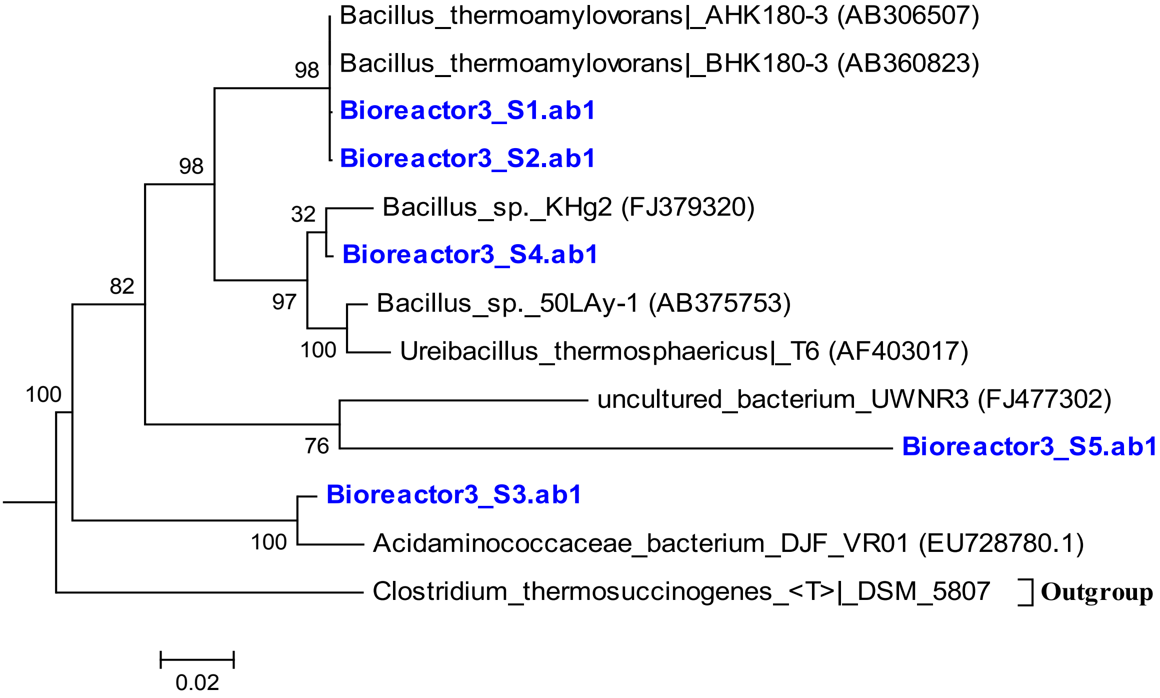


Figure 36: Phylogenetic tree derived from aligned 16S r DNA sequences of the excised DGGE bands (written in blue) of amplified 16S rDNA genes of bacterial consortium from Bioreactor 3 when the internal temperature of the bioreactor was 65°C. The tree is based on the Ribosomal Database project Jukes-Cantor distance model using Neighbor-joining algorithm with 100 bootstrapping. The scale bar represents 0.02 nucleotide divergence

The culturable isolate in Bioreactor 3 was *Bacillus thermoamylovorans*, this facultative anaerobe is capable of heterolactic fermentation of hexose with end products such as lactate, acetate, ethanol, formate, but no hydrogen (Combet-Blanc *et al*., 1995). However, when *B. thermoamylovorans* is co-cultured with a *Clostridium* microbe, the hydrogen- producing fermentation has a high efficiency, high stability and high reproducibility (Huang *et al*., 2009). *B. thermoamylovorans* in co-culture has decomposition enzyme activity, thus decomposing large molecules such as yeast powder and small molecules i.e nutrient substances to provide nutrients for the *Clostridium* or other co-culture strains (Huang *et al*., 2009). *B. thermoamylovorans* requires yeast for luxuriant growth (Combet-Blanc *et al*., 1995), which was the sole carbon source in the medium described in Section 2.3. It has a temperature optimum of 50°C with an upper limit temperature for growth at 58°C, however, it was isolated from the bioreactor with an operational temperature of 65°C (Combet-Blanc *et al*., 1995). *Bacillus* KHg2 was identified as the endospore forming bacterium, as it has the same morphological characteristics as *Bacillus silvestris* (Zeng *et al*., 2009; Wongwilaiwalin *et al*., 2010). Thus it is a Gram-positive, rod-shaped, round terminal endospore-forming bacterium (Rheims *et al*., 1991). It grows at temperature ranges of between 10°C and 40°C with an optimum temperature of between 20-30°C (Zeng *et al*., 2009). It doesn't grow at pH values above 5.7. It utilizes glycerol and ribose as the sole carbon source without acid production (Zeng *et al*., 2009; Wongwilaiwalin *et al*., 2010). It produces acid from sucrose hydrolysis after three days of incubation (Zeng *et al*., 2009). *Rhodocyclaceae* are a family of Gram-negative bacteria, that are mainly aerobic/facultative anaerobic which are denitrifying rod shaped bacteria (Zeng *et al*., 2009). This bacterial strain together with the other facultative anaerobes would consume available oxygen, thus triggering oxygen depletion in the system and allowing obligate anaerobes to grow (Zeng *et al*., 2009). Band analysis in the study by Wongiwilaiwalin *et al.* (2010) revealed that the *Rhodocyclaceae* bacterium was the major oxygen consuming bacteria in the thermophilic degradation of lignocellulose bacterial consortium (Wongiwilaiwalin *et al*., 2010). Some *Rhodocyclaceae* bacteria can utilize fermentation products such as acetate under microaerophillic or anaerobic conditions to produce 1.7mol H_2/mol acetic acid (Wei *et al*., 2010; Wongwilaiwalin *et al*., 2010). Acetate is one of the main by-products in glucose fermentation during biohydrogen production (Borole *et al*., 2009). Borole *et al.* (2009) found that 48.9% of the clones in a microbial fuel cell were *Rhodocyclaceae* (Borole *et al*., 2009). These were found to be closely affiliated to organisms capable of nitrate reduction. This allows for justification of the 26% H_2 content of the total gas present at 65°C in Bioreactor 3. Since the H_2 production system has hydrogen consumers (Borole *et al*., 2009).

The presence of *Acidaminococci*, that utilizes glutamate as the sole carbon source indicates that there has to be glutamate-producing microbes such as *Corynebacterium glutamicum* in Bioreactors 1 and 2 where sucrose was the sole carbon source (Georgi *et al*., 2005). *C. glutamicum* is a Gram- positive rod shaped bacteria that forms glutamate and L-lysine from several carbohydrates such as glucose, fructose and sucrose (Georgi *et al*., 2005). The Clostridial medium used in Bioreactor 3 had yeast powder as the sole carbon source. Yeast powder is rich in vitamins, minerals and digested nucleic acids and amino acids. According to Tanguler and Erten (2008) autolysed brewer's yeast has 3.9% of α- amino nitrogen and 45.6% protein (Tanguler and Erten, 2008). Since glutamate/glutamic acid is one of the 20 proteinogenic amino acids, the yeast powder is the possible source of the glutamate, thus encouraging *Acidaminococcal* growth.

CHAPTER 4: CONCLUSION

Hydrogen is seen as an acceptable environmentally safe and renewable energy source which can be an alternative for fossil fuels. Past research conducted on hydrogen produced by microorganisms has shown that the production of biohydrogen using sewage sludge and wastewaters as substrates also serves to remedy waste removal problems. According to the best of my knowledge, a highly efficient system with a high level of biohydrogen production has not yet been developed. This research aimed to identify microorganisms capable of biohydrogen production in order to manipulate environmental conditions to maximise substrate utilisation and hydrogen production.

Molecular identification of the H_2- producing bacteria is essential in order to manipulate bioreactor conditions in such a way that would yield the highest possible hydrogen yield. Given that the conditions of the AFBRs were such that inhibited the growth of methanogens and enteric bacteria, only fermenters and acetogenic hydrogen- producers were expected to be found during molecular identification. However, *Citrobacter* spp and *Shigella* spp were observed in Bioreactor 1 at 55° C, whereas *Klebsiella* spp and *E. coli* were observed in Bioreactor 2 at 55°C and Bioreactor 3 at 65°C. These could however be present as sample contamination. Enteric bacteria are facultative anaerobes which deplete oxygen from the systems, thus creating an anaerobic condition that promotes growth of obligate anaerobes. Bioreactor 1 had *Bacillus coagulans*, which produces 3mol H_2/ mol sugar, and *Citrobacter* as the hydrogen producers at 55°C. These produced 38% hydrogen content in the gas removed from the gas flow pipe. *Clostridium thermopalmarium* and *Acidaminococcus* as biohydrogen producers, which produce 4mol H_2/ g glucose and 1 mol H_2/ g glutamate, respectively. This contributed to the higher percentage of hydrogen of 55% in the system at 65°C.

Bioreactor 2 had *Paenibacillus* as the dominant bacteria, however this bacteria was not culturable on solid or liquid media. Since *Paenibacillus* it produces approximately 1.3mol ethanol/mol glucose, it could have possibly suppressed the growth of hydrogen producers by negatively affecting hydrogenase activity as accumulation of ethanol drops the pH of the system to values below 4.2. This would support the fact that no hydrogen producers were isolated in the bioreactor at this point. Three primary fermenters were present in Bioreactor 2, with two hydrogen producers *B. coagulans* and *Acidaminococcus*. *Actinomyces* was the primary fermenter at 65°C, fermenting sucrose to formate/ formic acid and formate serves as a substrate for hydrogen production. *B. thermacidophilum* produced acetic acid and lactic acid from glucose fementation. *Selemonas bovis* also served as a primary fermenter fermenting the sucrose into acetate and propionate. *Selemonas ruminantium* a secondary fermenter that uses succinate and lactate to propionate, acetate, CO_2 and for some strains trace amounts of H_2. *B. coagulans* was the culturable biohydrogen producer highest hydrogen yielding 3mol/ mol sugar when sucrose is the substrate (Kotay and Das, 2007). *Acidaminococcus* was the hydrogen producer which utilises glutamate/ glutamic acid. These only managed to produce 46% hydrogen content in the gas removed from the gas flow pipe. Bioreactor 3 had *Rhodobacterium* as the sole biohydrogen producing bacteria at 55°C. *Bacillus thermoamylovorans* was a culturable fermenter found at 65°C. It is heterolactic with the fermentation of hexose with end products such as lactate, acetate, ethanol and formate. These serve as substrates for hydrogen production. *Acidaminococcus* bacterium and Rhodocyclaceae were the biohydrogen producing bacteria isolated at 65°C resulting in a 26% hydrogen content of the air removed from the gas flow pipe.

The presence of Rhodobacteria in the system can be contributed to the yeast extract that provides a sufficient supply of nitrogen and phosphorus (Kotay and Das, 2010). Rhodobacteria are nitrogen fixating bacteria and require nitrogen for their preferred mode of growth, photoheterotrophic growth.

The constant temperature of 65°C and the longer hydraulic retention time of Bioreactor 1 allowed for *Clostridium* species to grow. An HRT range of 6.5-12 hours would promote the growth of *Clostridium* species, thus a steady production of biohydrogen (Chang *et al*., 2008). Wei *et al*. (2010) found that low HRT lead to a big loss in high efficient hydrogen- producing bacteria added to the system during bioaugmentation whereas an HRT of 6 hours allowed for survival of these high efficient biohydrogen producers in the system (Wei *et al*., 2010). The longer HRT allowed for the high hydrogen producing *C. thermopalmarium* to grow. This *Clostridium* was culturable, thus allowing for H_2-producing system enrichment and a good candidate for bioaugmentation. Bioaugmentation is when dominant bacterium genus isolated and selected from nature are directly added to the wastewater treatment system in order to improve capacity of the former treatment system and reach a target to eliminate harmful matter or optimize a property (Wei *et al*., 2010), in this case biohydrogen production and sewage sludge degradation.

The constant acidic pH of 5.5-6 and the constant thermophilic temperature allowed for the removal of pathogens and contaminating microbes from the system, favouring *Clostridium* growth. Since the generation of hydrogen by microorganisms is coupled with the formation of organic acids as metabolic products a constant pH range was desirable as the accumulation of acid would cause a sharp drop in culture pH (Nath and Das, 2004). Meyer (2007) showed that hydrogenase activity, which is required by anaerobic bacteria to further breakdown the organic acids to form hydrogen, is best at pH values higher than 5.2 (Nath and Das, 2004; Meyer, 2007). At pHs lower than 5.2 accumulation of metabolites occurs, thus suppressing H_2-producing bacteria (Nath and Das, 2004; Wang et al., 2006; Meyer, 2007).

For Bioreactor 2 the variable temperature resulted in various potential H_2- producers, however only *Bacillus coagulans* was culturable under anaerobic condition for enrichment purposes. The cow-dung inoculum resulted in facultative anaerobes as the predominant species and without the presence of major hydrogen producing species, with *Acidaminococcaceae*, Rhodobacterium and Rhodocyclaceae being the only potential H_2 producers isolated. However, no hydrogen producers were culturable. Therefore it can be concluded that sewage sludge is a better source of potential H_2- producing microorganisms than cow dung. Sewage sludge is an important renewable source which has a high nutrient content consisting typically of 41% protein, 25% lipid, 14 % carbohydrates and 20% unknown on the basis of COD (Carbon Oxygen Demand) (Kotay and Das, 2010).

Grant and Long (1981) studied sewage digestion and identified classes of anaerobic bacteria involved in sewage digestion phase 1, the non methanogenic phase where hydrogen is produced (Grant and Long, 1981).

The list included facultative anaerobes such as *Bacillus* spp, *Klebsiella* spp and *Actinomyces* spp. The obligate anaerobes such as *Clostridium* spp and *Veillonella* spp, a group under which *Acidaminococcus* spp and *Selenomonas* spp are classified (Grant and Long, 1981). All these classes were present in the isolates from the three AFBRs.

Since Bioreactor 2 had the largest diversity of bacteria at 65°C it was expected to have a higher hydrogen yield because diversity is generally associated with improved community function and stability (Kotay and Das, 2010). This is usually attributed to:

(i) More efficient utilization of resources due to increased competition, niche differentiation and resource exploitation and

(ii) The presence of individual species with crucial functional characteristics (Kotay and Das, 2010).

It seems, however, that community structural and functional characteristics contributed more than species diversity to the efficient production of hydrogen (Kotay and Das, 2010).

I believe that biohydrogen would be an ideal fuel of the future as it doesn't use human food sources as the starting material like bioethanol and biodiesel do. Bioethanol and biodiesel are made of corn and vegetable oil, respectively. Biohydrogen production uses a system that is already in place such as sewage sludge, which usually requires drying in order to use as fertilizer, as the source for microorganisms essential biodegradation of industrial effluents. Sewage sludge is readily available from domestic wastewater treatment facilities.

In the future a successful research undertaking will be one that uses industrial food processing effluents as the sole carbon source and uses untreated sewage sludge to produce biohydrogen and render the effluents as an environmentally friendly liquid.

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