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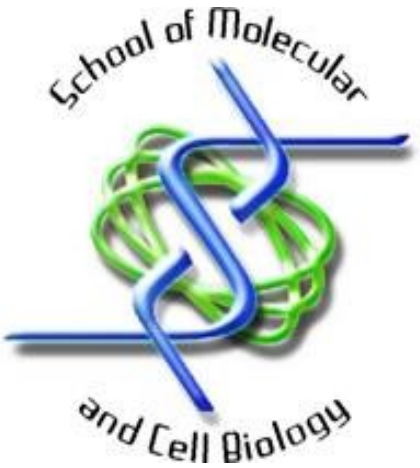

ANAEROBIC BIOHYDROGEN PRODUCTION BY A FLUIDIZED GRANULAR BED BIOREACTOR UNDER THERMOPHILIC CONDITION

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment for the Degree of Master of Science.

By

Phumlani Masilela

May, 2011

DECLARATION

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

Phumlani Masilela _____ _____

Signature Date

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I wish to especially thank my academic supervisor Prof V.M. Gray who provided with infrastructure and guidance throughout my research. Further thanks are given to my advisor Dr Togo for his advice. I am also grateful to my family and friends: Thabo Nkosi, Charity Nakana, Thalitha Nkomo, Lubanza Ngoma, Franklin Obazu and Musa Marimani for their support and endless love. My thanks are also extended to a long list of people including support staff within the school (MCB and Scanning Microscope Unit) whose continuous support and encouragement helped me throughout my project and my time at the University. This study was financially supported by the Commission of European Communities, Sixth Framework Program, Priority 6, and Sustainable Energy Systems (HYVOLUTION). Other financial assistance was provided by:

- National Research Foundation (NRF)
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- Biotechnology Bursary.

Phumlani Masilela

PUBLICATIONS AND PATENT FROM THIS DISSERTATION

- Patents for the anaerobic fluidized granular bed bioreactor (AFGB) system have been filed by Wits Enterprise Ltd, in the following countries
    - South Africa
    - European Union (EU)
    - United States of America (USA)
    - India
    - China

In our patent, gas disengagement and high rates of effluent recycling dilute H_2 sufficiently to promote complete oxidation of volatile fatty acids. This makes our approach very novel and unique.

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LIST OF ABBREVIATIONS

AFBR Anaerobic fluidized bed bioreactor

CAC Cylindrical activated carbon

CSTR Continous [flow] stirred tank reactor

DGGE Denaturing gradient gel electrophoresis

DNA Deoxyribonucleic acid

EPS Extracellular polymeric substance

GAC granular activated carbon

GC Gas chromatography

HRT Hydraulic retention time

HPR Hydrogen production rate (mmol h^{-1} L^{-1})

HY Hydrogen yield mol-H^2 mol-electron $donor^{-1}$

NAD^+ Nicotinamidenine dinucleotide (oxidized form)

NADH Nicotinamideadenine dinucleotide (reduced form)

ρH_2 Partial pressure of hydrogen

PCR Polymerase chain reaction

T Temperature (˚C)

UASB Upflow anaerobic sludge blanket

VFA Volatile fatty acids

ABSTRACT

There is now a critical need for development of full-scale practical application of fermentation technologies for energy generation (e.g. hydrogen production) that would be dependent on carbon neutral fuels such as biomass or wastewaters containing organic materials. Thermophilic fermentative biohydrogen production was studied in the anaerobic fluidized bed reactor (AFBR) operated at 65ºC with sucrose as a substrate. Theoretically, the maximum hydrogen yield (HY) is 4 mol H_2.mol^{-1} glucose when glucose is completely metabolized to acetate, H_2 and CO_2. But somehow, under most bioreactor design and operation conditions the maximum possible hydrogen yield (HY) has generally been observed not to exceed or reach 70-100% of the maximum theoretical hydrogen yield. In this study the application of external work in the form of high temperatures, high dilution rates and high rates of de-gassed effluent recycling were investigated as a means to overcome the thermodynamic constrains preventing the simultaneous achievement of high hydrogen yield (HY) and hydrogen productivity (HP) in an AFBR reactor. Bacterial granulation was successfully induced under a thermophilic temperature of 65 ^{o}C within a period ranging from 7 to 14 days. The bacterial granules consisted of a multispecies bacterial consortium comprised of thermophilic clostridial and enterobacter species. At a hydraulic retention time (HRT) of 1.67 h and effluent recycle rate of 3.5 L min^{-1}, hydrogen production rate (HPR) of 32.7 L H_2/h and hydrogen yield (HY) of 3.91 mol H_2/ mol glucose were achieved. The design and operation of our bench scale AFBR system has also resulted in HYs greater than 4 mol H_2/mol glucose. The maximum substrate conversion efficiency was 95%. However, it was noted that at very low HRTs (< 1h) the bioreactor substrate conversion efficiency dropped to 55%. This work demonstrated that the application of external work to a bioreactor in the form of high temperatures, high dilution rates and high rates of de-gassed effluent recycling could be used to overcome the thermodynamic constraints preventing the simultaneous achievement of high HYs and high HPs.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Traditional methods of energy generation

Human beings for many centuries have relied on fossil fuels for energy production to drive economic growth and industrialization- a correct choice at the time. However, we now understand better the negative impact of sourcing our primary energy from such a source. At present about 85% of world energy is derived from combustion of fossil fuels, for example nearly all of the commercial hydrogen (H_2) is produced from fossil fuels: 40% H_2 is produced from natural gas, 30% from heavy oils and naphta, 18% from coal, and 4% from electrolysis (Das, 2009). Combustion of fossil fuels during energy generation processes have many disadvantages, emission of carbon based pollutants into the atmosphere during combustion processes is the main cause of global warming, acid rain, and health problems (Das, 2009; Das and Verizoglu, 2001). In addition, fossil fuels are non-renewable energy resources and in the near future they will become depleted. Crude oil production will approach a theoretical depletion near 2060-2070, and theoretical depletion for natural gas is closer than for crude oil (Klaas, 2003).

1.2 Energy, environment, health and natural disasters

The world is faced with serious environmental problems, many due directly or indirectly to fossil fuel utilization. An estimated 40% of annual deaths are thought to be directly linked to environmental degradation (Pimentel et al., 2007) and poor air quality, largely due to fossil fuel combustion. It has been estimated that about 3 million people are killed worldwide each year by air pollutants (WHO, 2002), air-bone particulates emanating from vehicle exhaust, which are estimated to be responsible for 20% of the lung cancer deaths in the USA (Pearce, 2002). Fossil fuel driven climate change already has had an effect on human morbidity with conservatively, 150 000 deaths and over 5 million DALYs (disability adjusted life years) attributed to this factor (Campbell-Lendrum and Woodruff, 2007).

An energy crisis is looming and it is speculated that by 2050 energy demand will outstrip supply (Holmes and Jones, 2003). The current global energy consumption is approximately 500 EJ (1 EJ = 10^{18} Joules (J) = 10^{15} kilojoules (kJ) = 24 million tones of oil equivalent), projected world primary energy demand by 2050 is expected to be in the range of 600 to 1100 EJ (Energy Needs). The energy challenges is one of greatest test humankind has to face, with the threat of increasing in energy demand, depletion of carbon-containing fossil fuels and concerns over issues of environmental degradation.

1.3 Carbon credit and emission trading

In order to ensure that countries commit to emissions reduction, International treaties such as the Kyoto protocol set quotas on the amount of the greenhouse gases countries can produce over commitment period from 2008 to 2020. With the 1990 emissions as reference, Europe has agreed to an 8% emission reduction. Countries, in turn, set quotas on the emissions of business as set out by the Kyoto protocol (Lieffering et al., 2008). Since carbon dioxide and methane are the principal greenhouse gas, emissions trading are simple of trading in carbon credit. For trading purposes, one carbon credit is considered equivalent to one tonne of carbon dioxide emissions. Carbon is tracked and traded like any other commodity. In the "carbon market", certified emission reductions (CERs) can be exchanged between businesses or bought and sold in international markets at prevailing market prices.

Current median price in early 2008 for US carbon credits is around 6 US$ per metric ton, with projected median and high percentile prices increase to 13 and 27 US$ per metric ton, respectively by the end of 2012 when the current emission quota set by Kyoto protocol will be reviewed. Carbon emission trading has been steadily increasing in recent years. According to the World Bank's Carbon Finance Unit, 374 million metric tones of carbon dioxide equivalents were exchanged through projects in 2005, a 240% increase relative to 2004 at 100 million metric tones of carbon dioxide, which itself had a 41% increase relative to 2003 (78 million metric tones of carbon dioxide). In 2008, the world Bank, reported that a sharp rise in the number of transactions in the emissions trading market brought the value of trades to about $64bn in 2008 (World Bank, 2008).

1.4 Renewable energy technologies

Recently, there has been a surge of interest in CO_2-neutral clean energy and efficient energy generated from renewable sources. According to the European Commission's Renewable Energy Roadmap much efforts to reduce or replace consumption of fossil fuels has been underway in many countries, an objective to increase the share of renewable energies to 20% of gross inland energy consumption by 2020 was set out in the year 2007 (European commission, 2007). Within the renewable energy enterprise there is a great diversity of technologies and resources including "new" renewable energy sources such as photovoltaic power systems, wind energy systems, hydropower and biomass. Table 1.1 shows an overview of the leading resources and the technologies for harnessing them.

Table 1.1: Global renewable energy sources (Gross et al., 2003)

Resources	Scale of technical potential (usefull energy output) (TW h/year)	Energy conversion options
Direct solar	12,000 - 40,000	Photovoltaic Solar thermal power generation Solar water heaters
Wind	20,000 - 40,000 (onshore)	Large scale power generation Small scale power generation Water pumps
Wave	2000 - 4000	Numerous designs
Tidal	> 3500	Barrage Tidal stream
Geothermal	4000 - 40,000	Hot dry rock, hydrothermal, geopressed, magma (only hydrothermal currently viable)
Biomass	8000 - 20,000	Combustion, gasification, pyrolysis, digestion, for bio-fuels heat and electricity

Today, the potential use of renewable energy is great but on the other hand the contribution of renewables to world energy is still modest. Renewables covered only 13% of primary energy consumption globally in 2006, primarily through the use of wood as a fuel and hydropower (IEA Bioenergy, 2006), see figure 1.1.

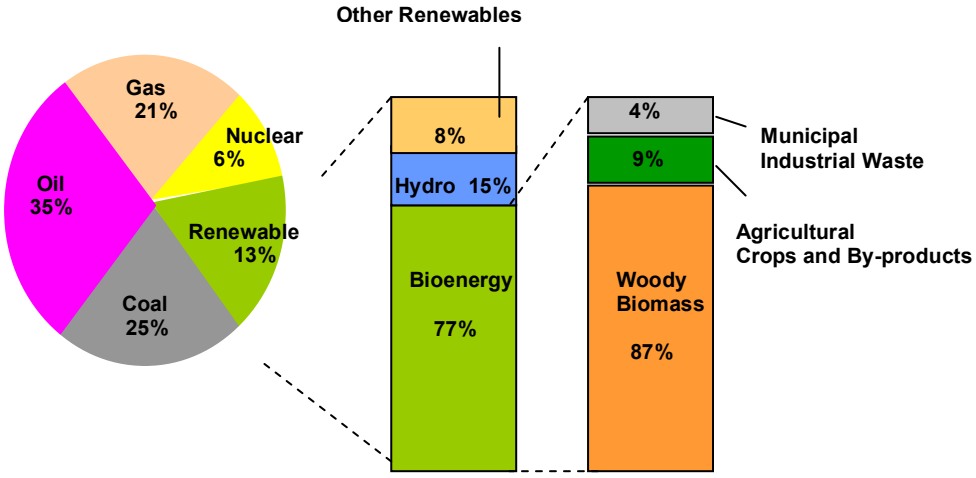

Figure 1.1: Share of bioenergy in the world primary energy mix. Source: based on IEA, 2006

There is a significant potential to expand biomass use for hydrogen generation by exploiting the large volumes of unused residues and wastes. The use of conventional crops for energy use can also be expanded, with careful consideration of land availability and food demand. The future energy economy will have an important role for hydrogen (H_2) as a clean, CO2-neutral energy source. Currently, H_2 is mostly generated from fossil fuels sources, in the long run, H_2 would preferably be produced by biotechnological processes. Biological hydrogen generating processes may provide a renewable, more sustainable alternative fuel to replace fossil fuels. However, Biological methods of hydrogen generation are yet to compete with those of commercial H_2 generating processes in terms of cost, efficiency and reliability (Das and Verizoglu, 2001).

1.5 Hydrogen (H_2)

Over the past two decades, H_2 has attracted an increasing attention around the world because it is an ideal energy carrier that is clean, recyclable and efficient (Das and Veziroglu, 2001; Brockris, 2002; Turner, 2004). H_2 is a colourless gas that accounts for about 75% of the universe mass. H_2 is found on Earth almost only in combination with other elements such as oxygen, carbon and nitrogen. Through the use of fuel cells H_2 can be used to generate electricity and heat at high efficiencies (Lay et al., 1999). H_2 is one of the most environmental friendly renewable energy sources, since the product of its combustion is water, see the below reaction 1.

$$H_2 + ½O_2 \rightarrow H_2O + 286\ kJ \ldots\ldots \text{(Reacrion 1)} \qquad \text{(da Rosa,2005)}$$

H_2 combustion has no contribution to environmental pollution and climate change (Levin et al., 2004). Therefore, hydrogen is expected to be a main energy fuel in the drive towards sustainable energy supply in the future. Figure 1.2 illustrate the history and the development of hydrogen energy. Brockris, who have contributed to the development of the concept of the "hydrogen economy", defines it as "the utilization of hydrogen to transport energy from renewable sources over large distances; and to store it (for supply to cities) in large amounts" (Brockris, 2002). Thus, the hydrogen economy includes the production, storage, distribution, and the use of hydrogen as carrier (Turner, 2004). The term hydrogen economy was developed in the early 1970s by technicians of General Motors (Brockris, 2002). However, the concept of hydrogen economy was developed far earlier (Dunn 2002; Turner, 2004). European Commission has outlined that hydrogen economy would aid in sustaining high life standard, and simultaneously providing a clean, safe, reliable and secure energy supply (European Commission, 2003b).

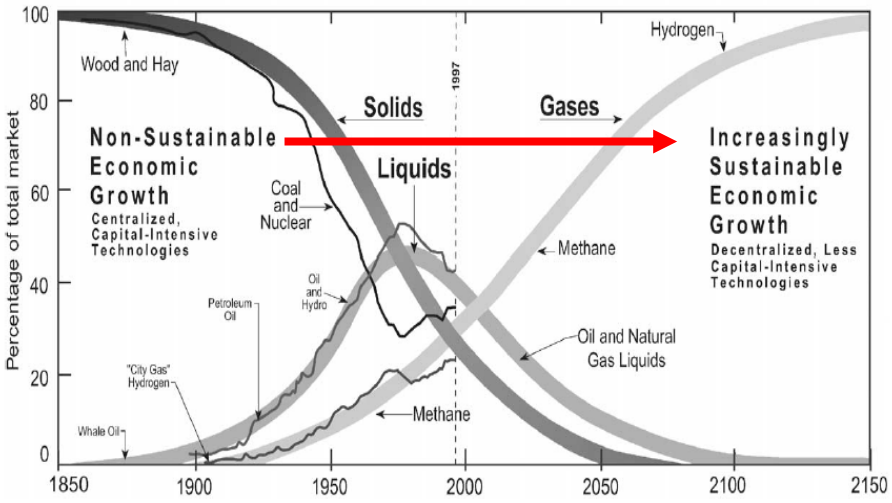

Figure 1.2: Graph showing the history and the future of energy supply economic development, Hydrogen Economy (Crabtree et al., 2004; Bossel et al., 2007).

1.5.1 Biological production technologies

Hydrogen production from biological systems is called biological hydrogen or biohyhdrogen (Kovacs et al., 2000). There are numerous attractive routes to produce biohydrogen from renewable source, currently known biological biohydrogen processes are shown in table 1.2. Mostly, biohydrogen producing methods utilizes microbes to produce biohydrogen from a wide variety of biomass substrates, including agricultural and forestry wastes, municipal solid wastes and animal wastes and residues (Carere et al., 2008; Muradov and Veziroglu, 2008). Some of listed biohydrogen generating methods in table 1.2 have drawbacks associated with them in terms of low hydrogen yield and production rate (Das and Veziroglu, 2008). Actually, great interest has been expressed towards dark hydrogen fermentation process, because it appears to be more favorable for biohydrogen production with concominant reduction in environmental pollutants, while other biological hydrogen generating methods listed in table 1.2, have low hydrogen production rates and yields as compared to dark fermentation process (van Ginkel and Logan, 2005; Levin et al., 2004). The purpose of biological hydrogen studies is to develop commercially practical hydrogen production processes by exploiting hydrogen producing ability of microorganisms through modern biotechnology. Attempts have already been made by several researchers to find out the suitability of different biological

processes, and in this study dark fermentation was studied to understand the present-state-of-art.

Table 1.2: Overview of currently known biological hydrogen production process (Beneman, 1996)

	Process		General reaction	Microorganisms used
1	Direct Biophotolysis		$2\ H_2O + light \rightarrow 2\ H_2 + O_2$	Microalgae
2	Photo-fermentations		$CH_3COOH + 2\ H_2O + light \rightarrow 4\ H_2 + 2\ CO_2$	Purple bacteria, Microalgae
3	Indirect biophotolysis	a	$6\ H_2O + 6\ CO_2 + light \rightarrow C_6H_{12}O_6 + 6\ O_2$	Microalgae, Cyanobacteria
		b	$C_6H_{12}O_6 + 2\ H_2O \rightarrow 4\ H_2 + 2\ CH_3COOH + 2\ CO_2$	
		c	$2\ CH_3COOH + 4\ H_2O + light \rightarrow 8\ H_2 + 4\ CO_2$	
			Overall reaction: $12\ H_2O + light \rightarrow 12\ H_2 + 6\ O_2$	
4	Water Gas Shift Reaction		$CO + H_2O \rightarrow CO_2 + H_2$	Fermentative bacteria, Photosynthetic bacteria
5	Two-Phase $H_2 + CH_4$ Fermentations	a	$C_6H_{12}O_6 + 2\ H_2O \rightarrow 4\ H_2 + 2\ CH_3COOH + 2\ CO_2$	Fermentative bacteria + Methanogenic bacteria
		b	$2\ CH_3COOH \rightarrow 2\ CH_4 + 2\ CO_2$	
6	High-yield Dark Fermentations		$C_6H_{12}O_6 + 6\ H_2O \rightarrow 12\ H_2 + 6\ CO_2$	Fermentative bacteria

CHAPTER TWO

LITERATURE REVIEW

2.1 Dark hydrogen fermentation

Dark fermentation is the process whereby carbohydrates-rich substrates are decomposed by different anaerobic bacteria to produce hydrogen (H_2) and carbon dioxide (CO_2) and/or methane (CH_4), and other products, such as acids (e.g. lactic acid, acetic acid, butyric acid, propionic acid…etc) and alcohol (ethanol, butanol, propanol). The process by which H_2 is formed involves a complex interaction of various microorganisms and takes place in basically four separate phases namely: hydrolysis (phase 1), acidogenesis (phase 2), acetogenesis (phase 3) and methanogenesis (phase 4), see figure 2.1.

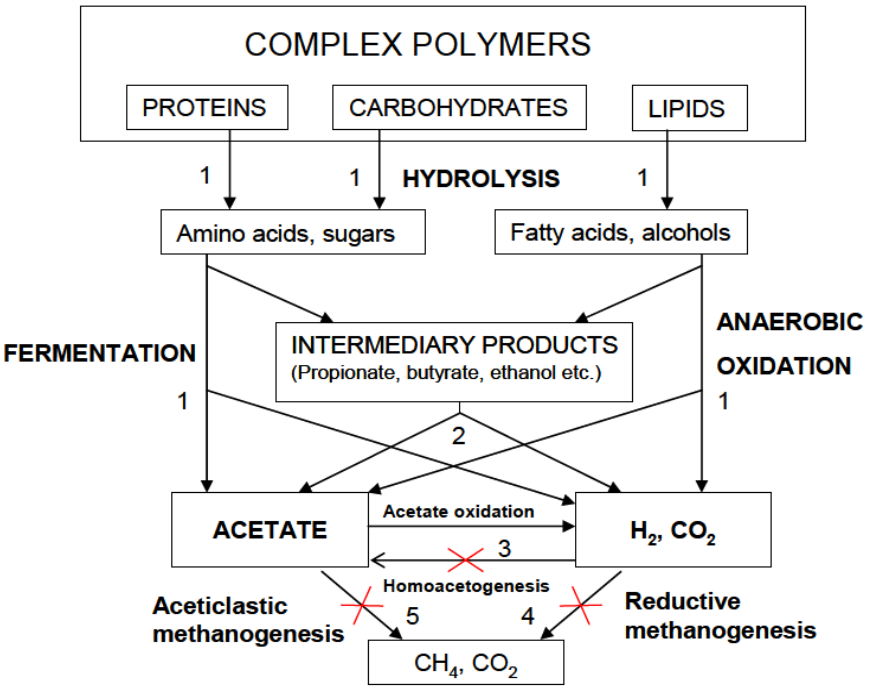

Figure 2.1: Different stages of anaerobic digestion of organic matter and the microbial groups involved.1, Fermentative bacteria; 2, hydrogen-producing acetogenic bacteria; 3, hydrogen-consuming acetogenic bacteria; 4, carbon dioxide-reducing methanogens; 5, Aceticlastcic methanogens (modified from Pavlosthathis and Giraldo-Gomez, 1991). The crosses represent hydrogen consuming reactions, methanogenesis and homoacetogenesis, which are undesirable in H_2 producing reactors.

2.1.1 Hydrolysis

Hydrolysis is the first step in anaerobic process whereby complex organic compounds (e.g. carbohydrates, proteins and lipids) are split into simpler components or simple monomers. These monomers which are the products of external hydrolytic reactions can be taken up across cell membranes and used as substrates for catabolism and anabolism. The breakdown of large biopolymers into the constituent monomers are catalysed by extracellular hydrolytic enzymes (cellulase, protease, lipase) released by facultative or obligate anaerobic bacteria (Gavrilescu, 2002).

2.1.2 Acidogenesis

Acidogenesis, also called fermentation is a process by which soluble molecules are used as carbon and energy sources by fermentative bacteria and converted into volatile fatty acids (VFAs), alcohols, and biogas (Reith et al., 2003). Acidogenesis is very important in anaerobic digestion as it is a step where H_2 is produced. H_2 comes from the mechanism of dehydrogenation of pyruvate by ferredoxin and NADH reductase enzymes and also from the conversion of formic acid by formate dehydrogenase. H_2 is one of the substrates from which methane (CH_4) is formed, as shown in figure 2.1, methanogenesis routes shown by red crosses should be avoided in fermentative hydrogen process. For acidogenesis to take place, some conditions such as nature of the culture, temperature, pH and H_2 partial pressure must be controlled to direct the process to the formation of expected end-products (Gavrilescu, 2002).

There are mainly four fermentation types in the anaerobic acidogenesis of organic matters (e.g. glucose), namely acetic acid fermentation, propionic acid type fermentation, butyric acid type fermentation and ethanol type fermentation. Most of microbial communities exhibit acetic acid fermentation with acetate acid as a major product (Datta, 1981; Chan and Holtzapple, 2003).

2.1.3 Acetogenesis

Acetogenesis is part of the fermentation process where more reduced compounds such as aromatic compounds, long VFAs and alcohols are converted to acetic acid and H_2 (Gavrilescu, 2002). VFAs such as acetate, propionate, butyrate, are mojor intermediate products in acidogenesis and acetogenesis stages of anaerobic biochemical degradation. The stability of over-all biochemical reactions relied on the degradation of VFA by anaerobes to the final gaseous products. Butyrate degradation differs from that of acetate as it includes acetogenesis step in the biochemical reactions, shown in reaction 2,

$$\text{Butyrate} + 2H_2O \rightarrow 2 \text{ acetate} + 2H_2 + H^+ \text{.....(Reaction 2)}$$

Conversion of butyrate to acetate is not thermodynamically favourable unless the acetate and hydrogen produced by the acetogens can be readily removed by acetotrophic and hydrogenotrophic bacteria, respectively (Gujer and Zehnderr, 1983). The conversion of acetate to hydrogen according to the reaction 3,

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + CO_2 \quad \Delta G^o = +104.6 \text{ kJ}\ldots\ldots \text{ (Reaction 3)}$$

is thermodynamically unfavorable at moderate temperatures (ΔG =+ 104.6 kJ mol^{-1}) and is strongly determined by the hydrogen partial pressure (Classen et al., 1999). For acetate oxidation to hydrogen the H_2 partial pressure must be kept very low by H_2 removal.

2.1.4 Methanogenesis

This process involves methanogenic bacteria which convert H_2 and acetate and CO_2 produced by the fermentation step to methane (CH_4). Methanogenesis is the final stage of the anaerobic digestion. Two groups of methanogenic microorganisms are involved: aceticlastic methanogenesis (Reaction 4) and hydrogenotrophic methanogenesis (Reaction 5), which involve hydrogen oxidation to methane (Handajani, 2004).

Aceticlastic methanogenesis

$$\text{Acetate} + H_2O \rightarrow CH_4 + HCO^-_3 \qquad \Delta G^o = -31 \text{ kJ/mol methane}\ldots\ldots\ldots\ldots \text{ (Reaction 4)}$$

Hydrogenotrophic methanogenesis

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 2H_2O \qquad \Delta G^o = -135.6 \text{ kJ/mol methane} \ldots. \text{ (Reaction 5)}$$

By using the mass balance the complete oxidation of glucose substrate to H_2, CO_2 and by products form, can be used to estimate the net hydrogen yield by each type of bacteria.

2.2 Substrate for dark H_2 fermentation

A wide range of Carbohydrates-rich substrates which can be used for the generation of hydrogen, includes feedstock's from energy crops (sugar beet, grasses, including lignocelluloses fractions), solid waste (food waste, organic fraction of municipal solid waste), and industrial wastewaters (food industries, pulp and paper industry). Due to global environment and energy security concerns, a non-polluting inexpensive feedstock must be used for hydrogen generation (Levin et al., 2007). Utilization of wastes to generate H_2 energy could reduce the production cost, making H_2 gas more available and cheaper.

2.3 Hydrogen producing micro-organisms: Mixed-cultures versus pure cultures?

During dark fermentative process, a variety of different microbes can be used to anaerobically breakdown carbohydrates-rich substrates to produce H_2 and other products, principally acids (lactic, acetic, butyric). H_2 producing microorganisms can be classified into two categories: facultative anaerobes (enteric bacteria, e.g. *Enterobacter and Citrobacter*) and strict anaerobes (*clostridia*). Enteric bacteria are rod-shaped, gram-negative facultative anaerobes, less sensitive to oxygen and are able to recover following air exposure (Nath and Das, 2004) the presence of oxygen, however, causes degradation of formate a major precursor for H_2 production, without H_2 formation. *Clostridia* are generally obligate anaerobes and are rod shaped with round or pointed ends in some cases. Rod shape can be either straight or slightly curve with 0.5-2 μm in diameter and up to 30 μm in length. *Clostridia* form a survival structure called endospores, which develops when the environmental conditions become unfavorable (high temperature, desiccation, carbon or nitrogen deficiency, chemical toxicity). When favorable conditions return, the spores germinate and become vegetative cells. These microorganisms can be classified into different functional groups according to their temperature tolerance as psychrophilic (13-18ºC), mesophilic (25-40ºC) or thermophilic (55-65ºC) (Metcalf and Eddy, 2003).

Cultures are selected either as single or mixed microbial strains. Some of the pure cultures known to produce H_2 from carbohydrates include anaerobes such as *Caldicellulosiruptor*, *Enterobacter aeruogens* (Rachman et al., 1998; Tanisho et al., 1987); *Ectothiorhodospira vacuolata* (Laurie and Roar, 1991) and *Citrobacter freunddeii, Citrobacter intermedius* (Oh et al., 2003). Pure cultures were cited as giving a relatively higher H_2 yield compared to mixed cultures (Wang et al., 2003). However, pure cultures are less useful for biohydrogen industrial applications because of the possibility of contamination and minimal sterilization. On the other hand, a mixed culture offers a superior bioreactor performance for industrial H_2 fermentation processes. H_2 production using cultures containing a mixed consortium of bacteria provides many advantages, the main one being that organic waste or waste water could be used without sterilization. This may confer large economic profits to the process. In addition, mixed cultures facilitate the co-existence of different kinds of cellulase systems for better cellulosic substrate degradation.

Mixed cultures can be obtained from natural resources, for example, rumen dung, soil and sewage sludge. These natural environments contain mostly *clostridia*. When mixed cultures from environmental sludge are involved in the anaerobic treatment process, an enrichment procedure for producing an inoculum suitable for biohyhdrogen production is necessary. In most cases, different methods have been used to select for H_2 producing communities and to inhibit the H_2-consuming bacteria, these methods include biokinetic (low pH and short hydraulic retention time) (Eun et al., 2004), heat-shock treatment (Setlow, 2003). According to a review by Kraemer and Bagley, (2007), heat-shock treatment has been the common method for killing methanogens (H_2-consuming microorganisms), leaving behind spore-geminating bacteria such as *Clostridium, Bacillus and Thermoanaerobacterium*. Some microbial species have the capacity to sporulate when environmental conditions become hostile such as heat-shock, changes in nutrient status, and among others presence of deleterious chemicals, and among others. The spores are metabolically dormant and resistant to heat, radiation, desiccation, pH extremes and toxic chemicals.

Recent advanced genetic engineering techniques have been suggested; if possible mixed microbial consortium can be design in order to create diverse members whereby each strain contributes a unique and essential metabolic capacity. This is due to the fact that in most studies microbial consortia vary overtime in the bioreactor, as shown by molecular (16S rDNA) studies (Lin et al., 2006). Nevertheless, it is necessary to establish mixed microbial consortia that can utilize diverse organic matter without sterilization and this may decrease the process costs.

2.4 Microbial identification techniques

Traditionally, microbes are identified by isolating individual cultures and examining their physiological, biochemical, and morphological characteristics. *Clostridium* was found using these methods as the H_2 producing bacterium in mixed culture (Lay, 2000). However, such identification is often unreliable and it is often difficult to culture most of bacteria from environment (Amann et al., 1995). First, microbes may not be properly isolated on artificial growth medium. Second, many microbes grow syntrophically with other species and thus cannot be cultured individually (Pike and Curds, 1971; Wagner et al., 1993). Third, many microbes share similar physiological, biochemical, and morphological characteristics and thus cannot be distinguished.

Recent advanced molecular techniques have been developed to analyze the structure and species composition of microbial populations. For instance, denaturing gradient gel electrophoresis (DGGE) separates polymerase chain reaction-amplified 16S ribosomal DNA (rDNA) fragments in polyacrylamide gels containing a linearly increased gradient of denaturants (Muyzer et al., 1993). DGGE has been used to identify complex microbial communities and to determine the phylogenic affiliation of community members (Ferris et al., 1996; Watanabe et al., 2000). DGGE analyzes PCR products of the 16S rDNA fragments which use a specialized primer that contains a GC rich region attached to the 5' end of the forward primer. The increasing gradient of denaturants allows for the separation of fragments based on their sequence differences. The 16S rDNA region is highly conserved in prokaryotes and is a stable part of the genetic code; hence the use of the 16S rDNA genes as a universal phylogenetic marker allows for the identification of the total microbial community in environmental samples. DGGE was originally

developed to detect specific mutations within genome genes due to one base mismatch (Myers et al., 1985). Since Muyzer et al., (1993) applied this method to environmental microorganisms, analysis of microbial communities using DGGE have become increasingly popular.

2.5 Biochemical pathways of hydrogen

The metabolic pathways implicated in fermentative H_2 production and the hydrogenase enzymes involved are well known and have been characterized in some details (Hellenbeck and Benemann, 2002; Hellenbeck, 2005, 2009; Lee et al., 2009). Figure 2.2 (a) describes the accepted metabolic steps in mixed acid glucose fermentation. Glucose is metabolized to pyruvate through glycolysis. Pyruvate is then converted into acetyl-CoA with the assistance of pyruvate-ferredoxin oxidoreductase. During pyruvate decarboxylation to acety-CoA, electrons move to ferredoxin (Fd) and finally end up in the production of protons, releasing H_2 (Saint-Amans et al., 2001; Thauer et al., 1977). Acetyl-CoA subsequently is involved in various fermentation pathways that ultimately generate alcohols (e.g. ethanol) and volatile fatty acids (e.g. acetate, butyrate, propionate, and lactate) (Carere et al., 2008; Desvaux, 2006; Nath and Das, 2004).

A complementary way for understanding interaction in complex mixed-culture systems is to track electron flow. An electron-flow study was performed in a pure-culture fermentation using electron equivalence (e^- equiv) balances and known pathways (Desvaux et al., 2001; Girbal et al., 1995a, b; Guedon et al., 1999a, b). Electron-flow model is based on two central principles. The first principle is that all e^- equivalence removed from substrate (e.g. glucose) must be accounted for in the fermentation products, such as H_2, acetate, butyrate, and ethanol. The second central principle is that the bacteria must balance $NADH_2$ production with $NADH_2$ consumption. $NADH_2$ is mainly produced during glycolysis in glucose fermentation. $NADH_2$ is consumed by the production of ethanol, butyrate, lactate, and propionate. Likewise, the electron carrier Fd_{red} must have equal production and consumption. Figure 2.2 (b) is a schematic diagram of the electron-flow model from glucose. The electron equivalence generated by glycolysis and pyruvate decarboxylation accumulates in the $NAD^+/NADH_2$ and Fd_{ox}/Fd_{red} pools, respectively. The reduced Fd is then oxidized by Fd-dependent

hydrogenase which transfers the electrons to protons resulting the formation of H_2. $NADH_2$ generated from glycolysis can be oxidized by $NADH_2$-Fd reductase in order to generate constant reducing equivalents for the catabolic process. Reducing equivalents can also be generated when $NADH_2$ is oxidized in the ethanol pathway and by lactate dehydrogenase (Lee et al., 2009; Carere et al., 2008; Desvaux, 2006; Nath and Das, 2004). When electrons of $NADH_2$ or Fd_{red} remain, these electrons can move between the $NAD^+/NADH_2$ and Fd_{ox}/Fd_{red} pools (dotted-line arrow, as shown in figure 2.2 (b)). The direction of this intra-electron flow depends on e^- equiv and H_2 relative to e^- equiv of Fd_{red} (Lee et al., 2009).

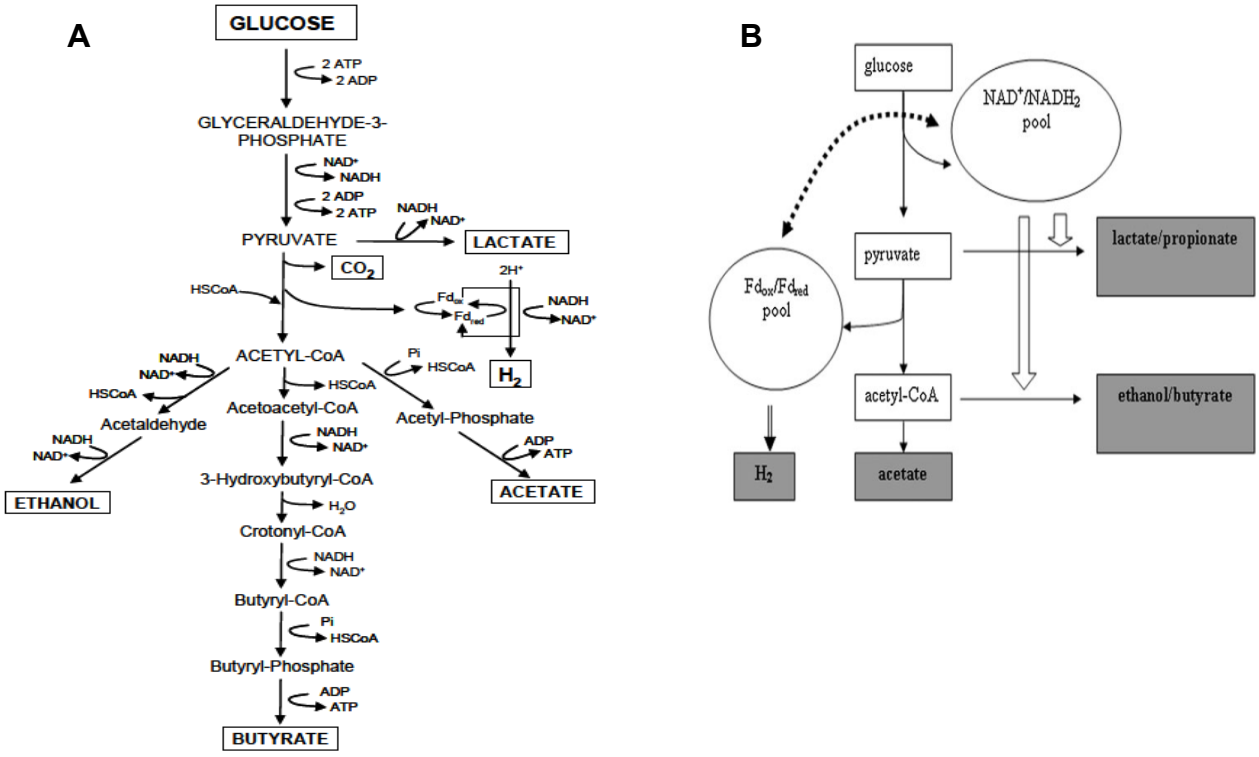

Figure 2.2: (a) A detailed proposed pathway of *Clostridium butyricum* (Modified from Saint-Amans et al., 2001; Cerere et al., 2008).(b) Schematic diagram of the electron-flow model. Electron equivalence are generated at glycolysis and pyruvate decarboxylation and accumulates as $NADH_2$ and Fd_{red}, respectively, in each electron carrier pool. Gray boxes are end products. The dotted arrow indicates electron flow between $NAD^+/NADH_2$ and F_{dox}/Fd_{red} pools. The dotted arrow indicates electron flow from Fd_{red} to proton, releasing H_2. Block-arrows indicate $NADH_2$ utilized for producing reduced liquid end products (lactate, propionate, ethanol and butyrate)(Lee et al., 2009) .

In fermentation processes there are butyrate/acetate and ethanol/acetate fermentation pathways involving H_2 production which involved the Fd: hydrogenase system. In comparison, formate is simply split to H_2 and CO_2 by formate-hydrogen lyase (Axley et al., 1990; Yoshida et al., 2006). Each H_2 type is associated with specific bacteria that have characteristics optimum conditions for H_2 production. *Clostridium sp.* usually are dominant H_2-producers via the butyrate/acetate fermentation pathway, and the optimal pH range is 5-6 (Fang et al., 2002; Fang et al., 2006a; Koskinen et al., 2007; Lee and Rittmann, 2009; Lee et al., 2008; van Ginkel and Logan, 2005a,b). *Ethanoligenes sp*. are abundant in ethanol/acetate fermentation, for which pH 4.5 is optimum (Ren et al., 2006, 2007). Fecal coliform bacteria (e.g*., Klebsiela sp., Escherichia coli, and Enterobacter sp*.) use the formate-cleavage pathway, and pH for the highest H_2 yield is around 7 (Nakashimada et al., 2002; Shin et al., 2007). In most cases the butyrate/acetate fermentation pathway seems to have the highest H_2 yield, up to 2.8 mol H_2/mol glucose (van Ginkel and Logan, 2005a).

2.6 The role of hydrogenases in biohyhdrogen metabolism

Among a large variety of micro-organisms capable of fermentative H_2 production, strict anaerobes such as members of the genus *Clostridium* have been most widely studied (Levin et al., 2004). *Clostridia* are dominant micro-organisms in mixed acid fermentation production of H_2 from biomass waste treatment. However, relatively little is known about the different forms of hydrogenases present in *clostridia*, and these enzymes have various physiological roles. There are three classes of enzymes which are capable of H_2 production: nitrogenases (Masukawa et al., 2002), alkaline phosphatases (Yang and Metcalf, 2004) and hydrogenase (Heinekey, 2009; Meyer, 2007; Vignais and Colbeu, 2004; Vignais et al., 2001). However, owing to their highly reactive and complex metallocentres, hydrogenases are regarded as the most efficient with turn over rates 1000 times higher than for nitrogenases (Hellenbeck and Benemann, 2002). They belong to an iron-sulfur (FeS) protein family that contains active sites consisting of inorganic sulfide and iron atoms bound by cysteinyl sulfur atoms to the peptide chain (Heinekey et al., 2009).

In most cases two basic metabolic types can be distinguished, in obligate and facultative bacteria, Fe-only hydrogenases and the [NiFe] hydrogenases. Fe-only hydrogenases seem to be restricted to strictly anaerobes, whereas [NiFe] hydrogenases are found more wide-spread in anaerobes, facultative anaerobes, and aerobes. Both types of hydrogenases play a key role in the fermentative production of H_2, by catalyzing the electron transfer reaction responsible for H_2 formation (Figure 2.3). During glucose fermentation pryruvate is oxidized to acetyl-CoA and subsequently to acetate, and different electron carriers can deliver the electrons to the terminal hydrogenase, viz. ferredoxin, NAD (H) or NADP (H), and these electron carriers are reduced in a limited number of oxidation steps in the central metabolic pathways. The two main oxidation steps during anaerobic sugar degradation (Embden-Meyerhof-Parnas pathway) are the conversion of glyceraldehyde-3-P to 3-P-glycerate and the conversion of pyruvate to acetyl-CoA.

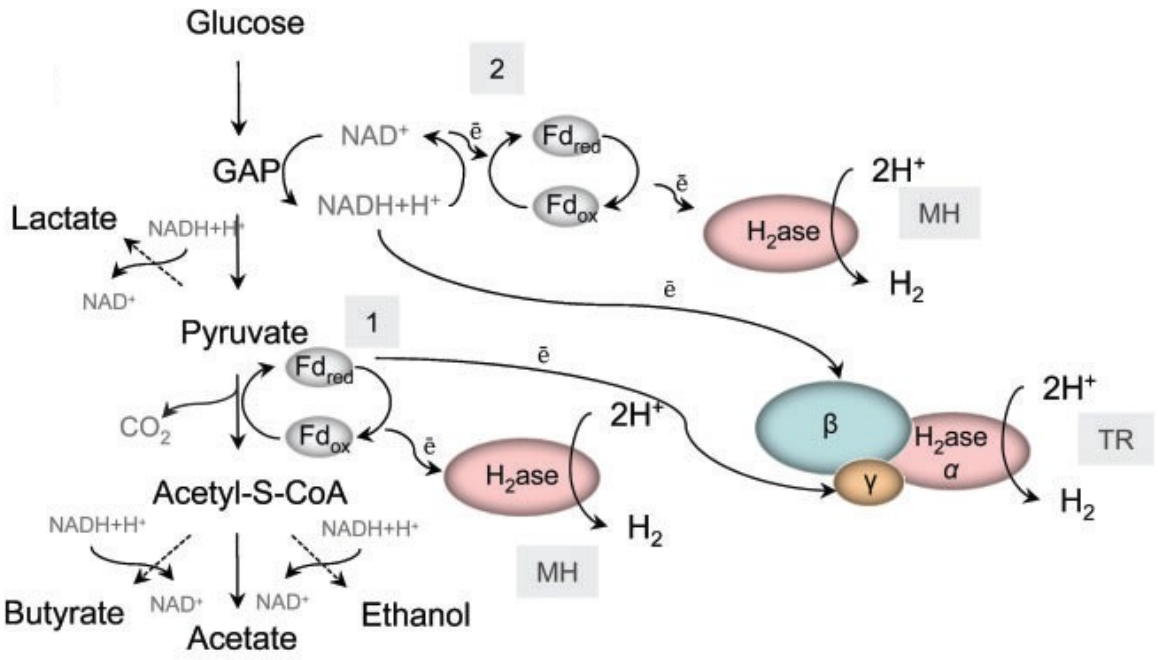

Figure 2.3: Fermentative hydrogen production pathway and the role of [FeFe] hydrogenases. There are two possible pathways for hydrogen production in clostridia. One is linked to the oxidation of reduce ferredoxin catalysed by the enzyme complex pyruvate: Fd oxidoreductase (1). The second involves ferredoxin-mediated NADH: Fd oxidoreductase (2) an alternative pathway involving trimeric bifurcating hydrogenase. Monomer hydrogenase; TH, Trimeric hydrogenase; Fd, ferredoxin; dashed lines, pathway competing for NADH + H+.

Hydrogenases, which are linked to the thermodynamically favored oxidation of reduced ferredoxin e.g. pyruvate:ferredoxin oxidoreductase, generating hydrogen by using proton as a terminal electron acceptors. A second pathway for hydrogen production is via NADH reoxidation during glycolysis, in which the cystolic hydrogenase, coupled to NADH:ferredoxin oxidoreductase, uses NADH as the electron donor to reduce protons to hydrogen (Vardar-Schara et al., 2007), and this activity has been demonstrated in many anaerobic fermentative bacteria including *Tt. maritime* (Schroder et al., 1994) and *Clostridium cellulolyticum* (Guedon et al., 1999).Hydrogen formation from NADH requires an NADH-dependent hyhdogenase, which has recently been characterized from *Ta tengongensis* (Soboh et al., 2004).

2.7 Thermodynamics of hydrogen formation

Thermodynamics plays an important role in chemistry, chemical engineering and in chemical process development. The use of thermodynamic methods for the predictions of the true yield and stoichiometry of bacterial reactions has been widely applied in biotechnology (Xiao and Briesen, 2005). However, these findings are sometimes very far from experimental results where many complicating factors include experimental errors, maintenance energy estimates, and simplifying assumptions, are present (Xiao and Briesen, 2008). Although as much as 12 mol H_2 can theoretically be derived from glucose, there is no known natural metabolic pathway that could provide this yield, due to the presence of other products (Woodward et al., 2000). Assuming that glucose is the substrate and acetic acid is the main product, the theoretical ratios of H_2 yield to substrate in a typical dark fermentation process may reach up to 4 moles of H_2 per mole of glucose utilized (See reaction 6), if the main aqueous product is butyrate only 2 mol of H_2 are produced (See reaction 7) (Rittmann, 2008).

Acetic acid: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$ .....(Reaction 6)

Butyric acid: $C_6H_{12}O_6 + 2H_2O \rightarrow CH_3(CH_2)_2COOH + 2CO_2 + 2H_2$…… (Reaction 7)

However, in a bacterial consortium there will be different microbial fermentation pathways, resulting in a mixture of products and the amount of H_2 generated will be determined by the acetate/butyrate ratio. In addition, the high partial pressure of hydrogen may result in metabolic shift towards the production of more reduced products (e.g. alcohols, lactate, butyrate, propionate etc) which will affect the final gas yield obtained (Bartacek et al., 2007; Nath and Das, 2004; Levin et al., 2004). It is clear that the H_2 production in fermentation associated with low H_2 yield is the result of large quantities of by-products formed. For optimal hydrogen yields formation of products like ethanol, lactate, propionate and others that consume hydrogen during their production must be avoided. Table 2.1 represents some of the metabolic reactions that bypass the major H_2-producing reactions in carbohydrate fermentation, and some of these reaction uses H_2 to form more reduced fermentation by-products.

Table 2.1: Biochemical reactions for formation of more reduced fermentation by-products, for simplicity, Gibbs free energy values are not shown

Fermentation reaction	Reaction
Propionic acid production with hydrogen	$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$
Ethanol production with hydrogen	$CH_3COOH + H_2 \rightarrow CH_3CH_2OH + H_2O$
Fermentation to ethanol	$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$

The H_2 yields and production rates of thermophilic bacteria, growing at temperatures above 60 ºC, often show higher values as compared to those of mesophilic bacteria. At elevated temperatures H_2 formation is thermodynamically more feasible and can produce up to 83-100 % of the theoretical maximum H_2 yield. This is due to the fact that an increase in temperature would enhance H_2 productivity and thermodynamic conditions which results in less undesired side products formation. These conditions, allows the bacteria to degrade acids to form H_2 and CO_2. Thermodynamically, acetate can be only oxidized to CO_2 at a very low hydrogen partial pressure (P (H_2)). Table 2.2 shows some of the thermodynamic favorable reactions which involves oxidation of volatile fatty acids

at elevated temperatures, provided *methanogens* are inhibited and when the hydrogen partial pressure is kept low.

Table 2.2: Biochemical reaction for oxidation of volatile fatty acids, for simplicity, Gibbs free energy values are not shown

Fermentation reaction	Reaction
Syntrophic propionic acid oxidation	$CH_3CH_2COOH + 2H_2O \rightarrow CH_3COOH + 3H_2 + CO_2$
Syntrophic butyric acid oxidation	$CH_3CH_2CH_2COOH + 2H_2O \rightarrow 2CH_3COOH + 2H_2$
Syntrophic acetic oxidation	$CH_3CH_2CH_2COOH + 2H_2O \rightarrow 4H_2 + 2CO_2$

2.8 Bacterial granulation technology

Granulation is a process whereby suspended bacterial consortia agglutinates either to themselves, or to suitable carrier particle or growth nuclei to form discrete well defined granules or biofilm (Liu et al., 2004; Tay et al., 2006). Anaerobic granules are characterized by their dense and strong microbial structure, regular, smooth round shape, ability to endure high flow rates and high organic loading rates (Liu and Tay, 2004). Granulation has been considered as the most effective means of ensuring biomass retention in hydrogen dark fermentation processes with biomass concentration of up to 79 gVSS/L reported in mesophilic systems (Lee et al., 2004b; Wu et al., 2006). Efficient cell retention enables high organic loading rates, and therefore, high H_2 production rates have been achieved with granular cell based reactors using mesophilic microorganisms. Hydrogen productivities up to 15.1 L/h/L for sucrose (Wu et al., 2006), 7.5 L/h/L for glucose (Zhang et al., 2008) have been obtained. Formation of bacterial granules in these reactors is a complex process, involving different trophic bacterial groups, and their physico-chemical and microbial structural interaction (Schmidt and Ahring, 1996; Zhang et al., 2007).

2.8.1 The first anaerobic granulation revolution

The concept of anaerobic treatment as the main biological step in wastewater treatment was rare until the development of the upflow anaerobic sludge bed or UASB reactors, discovered in 1970s (Lettinga et al., 1980). In UASB systems, formation of granular sludge (1-4mm) enhances the reactor biomass density and eventually promotes the efficiency in organic pollutant removal and methane production. A schematic of a classical UASB reactor is shown in figure 2.4. All the recent fluidized granular fluidized bed bioreactors are in reality modified versions of USAB bioreactors (Stronach et al., 1986).

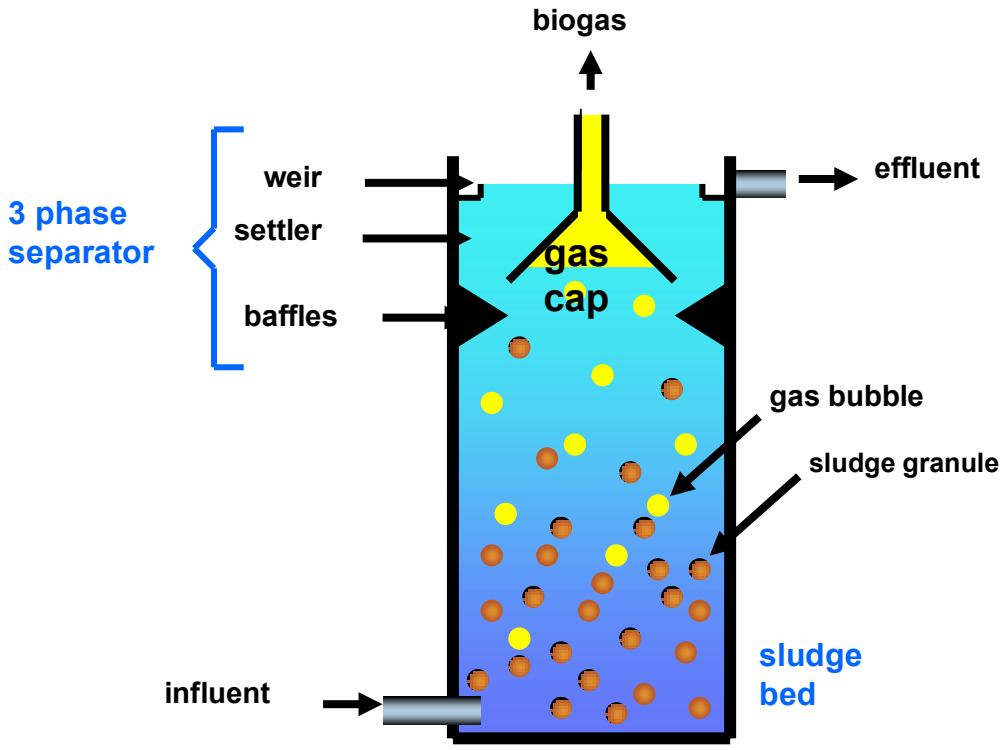

Figure 2.4: Schematic diagram of UASB reactor (Saravanan and Sreekrishnan, 2006).

2.8.2 Second anaerobic revolution

Recently, fluidized granular bed bioreactor or trickling bed bioreactors have been developed to initiate rapid induction, growth and development of bacterial granules. The most recent advance in granulation technology was made in 2004 (Lee et al., 2004). However, a major drawback is the long startup period for formation of granules, which sometimes requires several months (up to 6 months) to form granules (Fang et al., 2002; Mu and Yu, 2006; Mu et al., 2006; Oh et al., 2004, Yu and Mu, 2006).

A number of different or improved accelerated granule induction and growth protocols have been subsequently developed by various groups since 2004 (Lee et al., 2006; O-Thong et al., 2008; Zhang et al., 2007a; 2007b, 2008 b, 2008c). Zhang et al, (2008) reported that high H_2 production rates of 6.98 and 7.49 L/L/h were respectively achieved in both granule and biofilm based reactors. In their study they reported that granules formation was achieved within a period of five days by rapid approach of acid incubation (24h at pH 2) of bacterial culture (Zhang et al., 2007, 2008), while for others the granulation has been induced by using entrapped cells (Wu et al., 2006) or inert carriers (Lee et al., 2004a,b, 2006) and addition of cationic polymer (poly-acrylamide) and anionic organic material (silica sol), which can result in nearly immediate granulation (within 5 min; Kim et al., 2005). Furthermore, granulation can be induced by carrier matrices, cylindrical activated carbon (CAC) are normally packed at the bottom of the reactor to promote granulation (Thompson et al., 2008).

An example of an anaerobic fluidized granular bed bioreactor (AFBR) is shown in figure 2.5. The granular bed can be conceptualized as a stationary system through which the mobile bulk fluid phase moves at a velocity equal to the granule settling velocity. This phenomenon facilitates maximum mass transfer of both nutrients and gas molecules (H_2 and CO_2) between the mobile bulk phase and the fluidized *stationary* granular phase. The rate of H_2 removal from the granular bed is directly proportional to the volume flux of liquid phase through the bed. Bacterial granules have high biomass retention, robust structure and excellent settling properties (Tay et al., 2006), which allows the bioreactor

to be operated at relatively low hydraulic retention times (HRTs) or high dilution rates with minimal bacterial cell washout (Fang et al., 2002; Zhang et al., 2007c, 2008a).

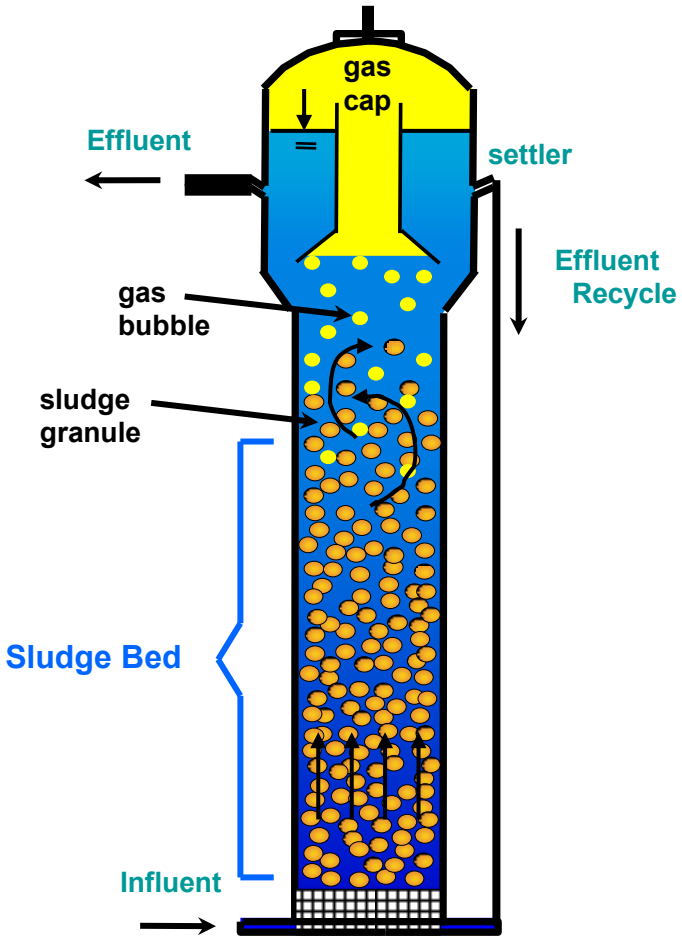

Figure 2.5: Anaerobic fluidized bed reactor (AFBR) (Saravanan and Sreekrishnan, 2006).

2.8.3 The importance of bacterial granulation in AFBR

The formation and the mechanisms of conventional granulation of anaerobic sludge in AFBR reactor have been well documented (Liu et al., 2002). Granules based reactors provide with improved bioreactor operational strategy and in most cases high hydrogen production rates can achieved in these reactors. This is due to the fact that granule-based reactors have compact and dense microbial structures which can withstand high effluent recycle rate (agitation), granules have excellent settling properties resulting in high

biomass retention, under high dilution rates with no significant bacterial washout occurring because granules have increased settling densities.

Recent advances in the capacity to initiate the rapid induction, growth and development of anaerobic bacterial granules and the application of bacterial granules in anaerobic bioreactors has allowed for the achievement of HPs greater than the 120 mol $H_2/(m^3.h)$ benchmark (Lee et al., 2006; Thompson et al., 2008; O-Thong et al., 2008; Zhang et al., 2007a, 2007b, 2008b, 2008c). Although this process of granulation has been largely studied, the mechanism of granulation formation has not been completely elucidated. It is therefore, important to further study and understand the mechanisms of bacterial adhesion, immobilization and development into mature granules in these bioreactors.

2.9 Factors affecting dark fermentative hydrogen production

In a fermentative H_2 production process, the major remaining stumbling block is incomplete substrate conversion, low H_2 production rates and the consequent low yields. In addition, H_2-fermenting microbes make other products to satisfy their metabolic needs and to further their growth; these include end products such as acetate, which permits ATP synthesis, and a variety of reduced products (for example, ethanol, butanol and butyric acids, lactic acid, propionic acid, etc). Types and relative proportion of products depends upon the organism, environmental conditions and the oxidation state of the substrate being degraded. Thus, H_2 fermentative production process is a very complex process and is influenced by many environmental factors such as temperature, hydraulic retention time (HRT), pH, and inoculum, substrates, H_2 partial pressure, reactor type, nitrogen, phosphate, metal ions, and the effect of these factors on fermentative H_2 production have been reported by a great number of studies throughout the world in the last few years (Fang et al., 2002; Tanisho et al., 1989; van Ginkel., et al., 2005; Das and Verizoglu, 2001, 2008; Hawkes et al.,2002, 2007; Kapdan and Kargi, 2006; Kraemer and Bagley, 2007). In this study a few of the above factors which have direct influence on the H_2 production process will be discussed and investigated.

2.9.1 Bioreactors for H_2 dark fermentations

For industrial scale application, the bioprocess technology especially the reactor would require a continuous production process with maximum steady state operation for longer periods of time and the bioreactor must demonstrate the ability to achieve high H_2 production rates and H_2 yields. In addition, the bioreactor system must allow good biomass mass transfer efficiency via vigorously mixing and also high biomass retention when the bioreactor is operated at high organic loading rates (i.e., low hydraulic retention time). Recent studies for H_2 production fermentations has been conducted in well-mixed immobilized-cell reactor systems includes continuous starred tank reactor (CSTR), carrier-induced granular sludge bed reactor (CIGSB), fluidized bed reactor (FBR), upflow-anaerbic sludge blanket (UASB) and granule-based continuous stirred tank reactor (CSTR). Overall, these immobilized-cell reactor systems are mainly based on the granulation process or biofilm attachment process, and relatively high unit volumetric H_2 production rates were found in these systems as a consequence of the elevated biomass retention (Kapdan and Kargi, 2006; Bartacek et al. 2007, Zhang at al., 2007, 2008).

Although the above mentioned reactors are excellent candidates for a practical H_2 producing process, operation at a very low HRTs (HRT<1h) still weakened the stability of the granular sludge bed, leading to washout of the H_2-producing sludge. Based on these considerations, much work is needed in order to improve the bioreactor performance in terms of bioreactor design that provide with maximum H_2 production rate and yields with the ability to maintain bacterial cell biomass even when operated at very low HRTs.

2.9.2 Hydrogen partial pressure (ρH_2)

Hydrogen partial pressure in the liquid is one of the key factors affecting H_2 production during the H_2 production process. In most anaerobic systems, the hydrogen mass transfer from liquid to gaseous phase is extremely limited and that the gaseous phase is not at thermodynamic equilibrium with the bulk liquid content of dissolved hydrogen. The quantity H_2 referred to as the actual dissolved H_2 may in fact consist of two components: solubilized H_2 and non-solubilized H_2. Non-solubilized H_2 consisting of H_2 molecules trapped in the liquid phase in the form of microscopic bubbles or aggregated clumps of H_2 molecules trapped within a matrix of H_2O molecules. Non-solubilized H_2 would be undergoing rapid dynamic reversible exchanges with solubilized H_2 resulting in a super-saturated equilibrium concentration of soluble H_2 in the liquid phase within the digester or bioreactor. The actual dissolved H_2 concentration in some anaerobic degradation process can be as much as 80-fold higher than the equilibrium value calculated from the hydrogen partial pressure in the gas phase (Kuroda et al., 1991). The liquid-gas transfer of H_2 should be driven from the liquid to the gas phase as fast as possible to maintain a high flux of H_2 production by the cell.

Efficient anaerobic degradation may be completed only under low levels of dissolved H_2 in the liquid surrounding the microorganisms, van Niel et al., (2003) reported that H_2 production by thermophilic bacterium was inhibited at H_2 pressures above 20 kPa, and a metabolic shift to lactate production was observed. It is well known that dissolved H_2 has proven to be an interesting parameter for reactor monitoring by showing a good correlation with short chain volatile fatty acid concentration, namely propionate, acetate, lactate and butyrate.

For example, the conversion of acetate to H_2 according to the reaction 8,

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + CO_2 \text{ …… (Reaction 8)}$$

is thermodynamically unfavorable at the moderate temperature ($\Delta G_0 = +104.6$ kJ mol^{-1}) and is strongly determined by the hydrogen partial pressure (Classen et al., 1999). However, if the digestion process of acetic acid is facilitated by the provision of an

additional supplement of thermal energy and also if the hydrogen partial pressure is kept at sufficiently low (<20 Pa) levels, then from the thermodynamic perspective it might be possible to push the above reaction in the forward direction. It is therefore important to monitor and decrease dissolved H_2 in the bioreactor bed. The decrease of H_2 partial pressure in fermentative H_2 process is considered as one of the approaches towards an improvement of H_2 productivity (Kumar and Das, 2001). There are several strategies which have been used to maintain hydrogen partial pressure within the desired range, hydrogen can be removed by stripping with inert gas, mainly nitrogen (N_2), and also H_2 partial pressures within the reactor can be lowered by increased bioreactor agitation, this is normally facilitated by rapid removal of H_2 as it is produced within the reactor (Mizuno et al., 2000).

2.9.3 pH

pH is another important factor that influences the activities of H_2 producing bacteria, and the fermentative H_2 production, pH has the direct effect on the activity of the hydrogenase enzyme as well as the metabolism pathway for H_2 generation (Wang and Wan, 2009). In addition, pH also plays a significant role on the bacterial surface physicochemical characteristics by influencing the bacterial electrostatic environment. The mechanism of pH change to accelerate the immobilization process of *clostridia*-like bacteria is unclear. However, several studies have mentioned that acidification stimulate the growth of H_2-producing bacteria, or improves adhesive properties of cells, thus resulting in less cell detachment occurring on immobilized particles (Zhang et al., 2007; 2008; Kraemer and Bagley, 2007). Several studies have reported that the pH 4.5 and below could negatively impact hydrogenase activity. In general, hydrogenase activity is low when the cell is maintained at a pH<5.2. Furthermore, Ren et al., (2007, 1997) demonstrated that when the pH in the fermentation system dropped below 4.5, ethanol could be produced during fermentative H_2 production. The accumulation of soluble metabolites (ethanol, propionate, lactate, etc), could suppress the activity of the H_2 producing bacteria and decrease the H_2 production rate and H_2 yield (Wang et al., 2006), thus is not favorable for the fermentative H_2 production.

According to a review by Wang and Wan, (2009), they reported that there exist some certain disagreements on the optimal pH for fermentative hydrogen production in both batch and continuous experiments, some of the studies with pH disagreement includes: Khanal et al., (2004) was 4.5; Lee et al., (2002) was 9.0; Mu et al., (2006) was 4.2; Zhao and Mu, (2008) was 7.0. The possible reason for this disagreement was the difference among these studies in the terms of inoculum, substrate and pH range studies. It is therefore recommended to further investigate the effect of pH on fermentative H_2 production.

2.9.4 Hydraulic retention time (HRT)

Most of dark hydrogen fermentation has been conducted in well mixed system, such as CSTR, ICSAB, ASBR, MBR, UASBAGSB and AFBR reactors and the most effective carbon substrate for H_2 production is carbohydrates (e.g. glucose, sucrose or starch)(Lin et al., 2008). In these systems there is a strong correlation between the systemic reduction in HRTs and the hydrogen production rate, whereby volumetric H_2 production tend to increase as the HRT decreases (Zhang et al., 2007, 2008; Lee et al., 2004). However, when the bioreactors are operated at high dilution rate (or low HRT) usually leads to washout of bacterial biomass causing a severe operational instability and inefficient H_2 production. Therefore, it is pivotal to retain high microbial biomass when the bioreactor is operated at low HRTs, and this could provide efficient H_2 production rate. Several strategies have been proposed to enhance biomass retention for high H_2 production, and these include strategies such as cell immobilization or granular sludge system. In addition, operating the bioreactor at low HRTs provides the following advantages: granule formation is facilitated at low HRT (Lee et al., 2004); *methanogens* can be suppressed by low HRT (Hawkes et al., 2007). *Methanogens* are slow growing micro-organisms, at high bioreactor dilution rates *methanogens* are washed out from the reactor system.

2.9.5 Temperature

Temperature is one of the most important factor that affect the activity of H_2 producing bacteria by influencing the activity of some essential enzymes such as hydrogenases for fermentative hydrogen production (Wang and Wan, 2009). A change in fermentative system temperature or a negative effect on hydrogenase enzymes might alter the substrate utilization process efficiency, H_2 productivity, liquid product distribution or microbial community (Fang and Liu, 2002). The effect of temperature on volumetric H_2 production rate (VHPR) can be explained thermodynamically by considering the changes in Gibbs free energy and in standard enthalpy of the conversion of glucose to acetate and assuming a maximum theoretical yield of 4 mol H_2 per mol glucose (Vazquez-Duhalt, 2002):

$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$.....(Reaction 9)

ΔG^o = - 176.1 kJ/mol

ΔH^o = + 90.69 kJ/mol

The above reaction based on the changes in the Gibbs free energy and enthalpy of the reaction indicates that the reaction can occur spontaneously and the reaction is endothermic, requiring heat energy to progress. The Van't Hoff equation can be used to explain the effect of the temperature on the equilibrium constant (Smith et al., 2000).

$$\ln\frac{K_1}{K_2} = -\frac{\Delta H^\circ}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) \qquad \text{....Equation 1}$$

$$\frac{K_1}{K_2} = \frac{[H_2]_1^4[CO_2]_1^2[CH_3COOH]_1^2}{[H_2]_2^4[CO_2]_2^2[CH_3COOH]_2^2} \qquad \text{....Equation 2}$$

If temperature increases, the equilibrium kinetic constant also increases because the reaction is endothermic (ΔH^o has a positive sign, see Eq. 1). Therefore, increasing temperature of the glucose fermentation, maintaining reactants concentration constant (see Eq. 2) would enhance H_2 concentration. Secondly, based on the thermodynamic models, it has been shown that high concentration of fatty acids may be better digested to

H_2 by the supply of thermal energy (e.g. thermophilic anaerobic digesters) (Kudora et al., 1991), for example, the conversion of acetate to H_2 according to the reaction: $CH_3COOH + 2H_2O \rightarrow 4H_2 + CO_2$, under low partial pressure.

The above mentioned thermodynamic considerations were previously supported, one of them, Veldez-Vazquez et al., (2005) studied the semi continuous H_2 production at mesophilic and thermophilic conditions. They found that VHPR was 60% greater at thermophilic than at mesophilic conditions. The authors suggested that this behavior may be explained by the optimum temperature for the enzyme hydrogenase (50 and 70ºC) present in thermophilic *Clostridia*.

In addition, thermophilic fermentation provide better conditions for inhibition of *methanogenic* bacteria (Lin et al., 2006; Kim et al., 2005; Lay et al., 1999), H_2 yield and production rates of thermophilic bacteria, growing at temperature above 60ºC, often show higher values as compared to those of mesophilic bacteria growing at moderate temperatures (Schonheit and Schafer, 1995). Nevertheless, there are specific constrains for H_2 production by thermophiles and (extreme) thermophiles, one of them is associated with low bacterial cell densities, which result in rather moderate H_2 productivities. In generally, there are very few studies dealing with sophisticated bioreactor systems for thermophilic H_2 production, it is therefore recommended that much more research is needed in order to fully understand the mechanism of hydrogen production under thermophilic conditions.

2.10 Approaches and theoretical considerations for maximum hydrogen production and yield

The recent flood of reviews on biohydrogen production is an indication that the discipline has now entered or even gone beyond the mature phase of development (Das, 2007; Davila-Vazquez et al., 2008; Hallenbeck, 2009; Hallenbeck and Gosh, 2009; Hawkes et al., 2007; Liu et al., 2008; Tsyganov, 2007; Valdez-Vazquez et al., 2009). An attempt to improve both the HP and HY in dark anaerobic processes appears to have now also reached the point of diminishing returns (Rittmann, 2008). Under most bioreactor design and operation conditions the maximum possible hydrogen yield in the anaerobic oxidation of glucose to acetate, H_2 and CO_2 has generally been observed not to exceed 4 mol H_2.mol^{-1} glucose, see equation 10 below:

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2 \quad \Delta G^{o'} = -206.1 \text{ kJ/mol}$$

...(reaction 10)

Given the strongly negative $\Delta G^{o'}$ for the above reaction, it seems possible that of the 24 electron equivalents (e^- eq) of glucose, 8 e^- eq should end up in H_2 with the remaining 16 e^- eq going to acetate. Because of internal bioreactor thermodynamic constraints dark fermentation hydrogen yields are usually below 4 mol H_2 /mol glucose (Rittmann, 2008). Theoretically acetate could be further oxidized under anaerobic conditions to yield 4 H_2 and 2 CO_2 in the absence of methanogens if the partial pressure of H_2 in the bioreactor can be reduced. Whether or not a practically viable anaerobic single or multi-stage bioprocess could be engineered, possibly with the application of external work in one form or another that would remove the potential energy barriers preventing the complete oxidation of glucose to 12 H_2 remains an interesting, but controversial consideration (Hallenbeck, 2009; Hallenbeck and Gosh, 2009), see below reaction .

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2 \qquad \Delta G^o = -25 \text{ kJ}$$

.....(reaction 11)

It is of critical importance to note that in practice HY values equal to or exceeding 3 mol H_2 / mol glucose have been attained only in situations where hydrogen productivity (HP) values have been low. Conditions that favor high HYs but usually result in low HP values

can be summarized as follows: thermophilic temperatures, low substrate loading rates, low dilution rates, low hydrogen partial pressures and low bacterial biomass densities. In addition, H_2 gas stripping by sparging with N_2 has been a necessary precondition for the achievement of HYs equal to or greater than 3 mol H_2. mol^{-1} glucose. Conditions that promote high HPs but usually result in low HYs values include the following: high substrate loading rates, high dilution rates, and high bacterial biomass densities. Operational conditions that favor high HPs also promote the maintenance of high hydrogen partial pressures within the bioreactor environment which in turn do not favor the attainment of HYs equal to or greater than 3 mol H_2 /mol glucose. In general the conditions promoting high HPs do not simultaneously favor the achievement of high HYs. Recently published surveys show that less than 5% of all reported HY values from a wide diversity of experiments were equal to or greater than 3.0 mol H_2. mol^{-1} glucose (Das, 2009; Davila-Vazquez et al., 2007; Wang and Wan, 2009).

2.11 The main aim of this research

This research was focused on the development of dark fermentative biohydrogen production using mixed bacterial cultures in a thermophilic (65°C) fluidized bacterial granular bed bioreactor (AFBR), that facilitates maximum hydrogen production and productivity. Sucrose was chosen as the sucrose substrate.

2.11.1 Specific objectives of this research:

- The first phase of this research was to develop a bioreactor design and operational strategy that facilitates maximum H_2 production and productivity.

- To develop a suitable procedure for rapid initiation, growth and development of thermophilic granules that consists of mixed hydrogen-producing microorganisms in the AFBR.

- To study the physical and physicochemical characteristics of the H_2 producing granules using both scanning electron microscope (SEM) and light dissecting microscope.

- To study the microbial diversity of the hydrogen-producing bacteria in the bioreactor using the 16S rDNA-based techniques (Polymerase chain reaction with denaturing gradient gel electrophoresis, PCR-DGGE).

- Investigate the effect of shortening the hydraulic retention time (HRT), increasing effluent recycle rates and pH on the substrate utilization, hydrogen content, hydrogen production, hydrogen productivity and the distribution of soluble metabolites in the AFBR.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Bioreactor nutrient medium formulation

A modified Endo medium formulation (Endo et al., 1982) was used as the bioreactor influent medium in this study. The modification involved the reduction in the concentration of sodium bicarbonate from 6.72 g L^{-1} to 3. 36 g L^{-1}. The inorganic minerals of the medium consisted (g L^{-1}): NH_4HCO_3 3.490; MnSO4 $CaCl_2$ 0.2; K_2HPO_4 0.699; $NaHCO_3$ 3.36; $MgCl_2$ $6H_2O$ 0.015; $FeSO_4$ $7H_2O$ 0.0225; $CuSO_4$ $5H_2O$ 0.005; and $CoCl_2$ H_2O 1.24 x 10^{-4}. The medium was supplemented with 17.63 g sucrose L^{-1}, (equivalent to 20 g COD L^{-1}).

3.2 Inoculum collection and preparation

An anaerobic thermophilic bacterial consortium was derived from a mixture of sewage sludge and fresh wet cow manure. Sewage sludge was collected from an anaerobic sludge digester at Olifansflei municipal wastewater treatment works (Johannesburg, South Africa). Fresh cow dung was collected from grass-fed cattle at Tembisa Township, east of Johannesburg. Collected cow dung and sewage sludge samples were mixed in a 500 ml Schott bottles. The inoculum mixture was pre-conditioned by acid and heat-shock treatment to enrich or select for anaerobic thermophilic hydrogen producing bacteria. Acid treatment involved lowering the pH of the inoculum mixture to 2 with 1 M HCL and incubating at pH 2.0 for 24 h at room temperature to inhibit the activity of the methanogens. Following the acid treatment, the pH of inoculum mixture was adjusted to 7.0 by mixing 50% v/v with Endo medium before heating at 90 °C in a water bath for 2 hours to remove non-sporulating hydrogen-consuming microorganisms, such as methanogenic microorganisms.

3.2.1 Inoculum Sub-culturing

After the acid and heat treatments 250 ml samples of inoculum mixture was inoculated into 1 L Schott bottles containing 250 ml Endo medium and incubated at 65 °C in a shaking incubator (3081U, labcon) set at rpm 86. Cultures were maintained by subculturing into fresh Endo medium every 2 to 3 days.

3.3 Anaerobic fluidized bed bioreactor (AFBR) design and set-up

The schematic diagram of the AFBR used in this study, is shown in figure 3.1. The anaerobic fluidized bed bioreactor (AFBR) was constructed from Clear Perspex and PVC materials. The three interconnected components making up the bioreactor consisted of a nutrient influent and recycled effluent inlet manifold, the main bioreactor bed (internal diameter (ID): 80 mm; height (H): 1000 mm) containing the fluidized bacterial granules and above the bed a solid-liquid separator (ID: 140 mm an H: 200 mm) to facilitate retention of granules by preventing washout of particulate biomass. The inlet manifold (ID: 80 mm and H: 150 mm) machined from solid PVC contained a hollow conical shaped diffuser which functioned at the primary inlet for the effluent recycle stream. A stainless steel sieve (32 mesh) was fixed over the inlet of the diffuser. Above the stainless steel sieve the conical diffuser was filled with a 100 mm layer of glass beads. Positioned above the hollow cone of the diffuser were 4 nutrient influent inlet ports (ID 5 mm) with each inlet arranged at 90° with respect to the two other inlet on each side. Nutrient medium (influent stream) was supplied directly into the upper glass bead layer via the 4 inlet ports. For the degassing of the effluent the bioreactor effluent overflow from the solid-liquid separator was decanted into an effluent gas-disengager. The effluent gas-disengager consisted of two components, a gas collection cylinder (H: 200 mm and ID 150 mm) with a gas outlet port and a gas-disengager cylinder (H: 600 mm and ID: 60 mm). The gas-disengager had two effluent outlets, one at the bottom that was connected to a variable Boyser® Bonfiglioli AMP-16 peristaltic pump (0.37 kW) which was used to recycle de-gassed effluent into the bioreactor via the diffuser. For effluent recycling the pump was set rpm at 45 rpm which gave a volumetric pumping rate of 3.2 L/min. The second effluent outlet drained the effluent overflow from the gas-disengager. Gas outlet

ports from the bioreactor and the gas disengager were connected via a Y junction to a gas meter (Ritter drum-type gas meter TG 05/3). The working volume of the bioreactor bed was 5.027 L. liquid-gas separator or gas-disengager had working volume of 1.54 L and the total fluid occupied volume of interconnecting piping was 0.934 L. Total fluid containing working volume of bioreactor system (bioreactor bed, gas-disengager, diffuser, and piping) was 7.501 L. Bioreactor and gas-disengager temperatures maintained at the operational temperature 65 ºC by circulating heated water from a heated water bath through the bioreactor and gas-disengager water jackets. Watson-Mallow Bredel (model 520U) peristaltic pumps (Falmouth, UK) was used to pump the Endo nutrient into the bioreactor.

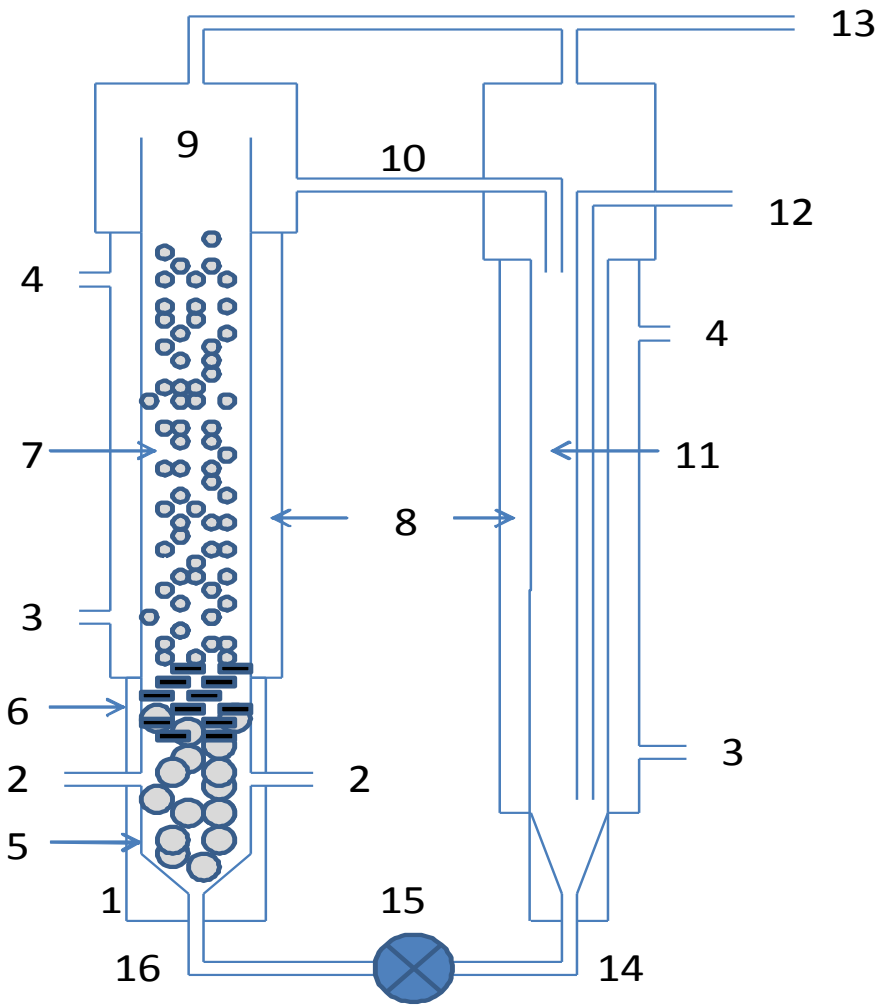

Figure 3.1: Schematic diagram of the anaerobic fluidized bed reactor used in this study. Diagram labels**:** 1 – inlet manifold; 2 – influent inlets; 3 – water jacket inlet for heat exchanger; 4 – water jacket outlet for heat exchanger; 5 – bed of glass bed (5 mm) in effluent/influent diffusion and cavitation generation; 6 – activated carbon for inducing granulation; 7 – fluidized bacterial granular bed; 8 – water jacket for heater 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.

3.3.1 Bioreactor startup, operation and bacterial granule induction

Cylindrical activated carbon (CAC) particles with the diameter of 2.5 mm and average length of 5.0 mm was used to facilitate the induction of bacterial granulation in the bioreactor. (Lee et al., 2004; Thompson et al., 2008). Prior to its use, the CAC was first washed with distilled water to remove all suspended fine particles and then sterilized by autoclaving for 20 minutes at 121 ºC. Sufficient CAC was added into the bioreactor to give a settled bed of 100 mm. Endo medium (5.0 L) and seed inoculum (2.0 L) was added to the bioreactor system. Seed inoculum consisted of an overnight culture. Following inoculation the bioreactor was operated on batch effluent recycle mode for 48 h to acclimatize the bacteria and allow for their attachment to the CAC. After this acclimatization period the bioreactor operation was switched to continuous effluent recycle mode with an initial HRT 10 h. The HRT was then decreased by increasing the nutrient medium supply rate. As the HRT was decreased from 10 to 4 h the growth and development of bacterial biofilms on the carrier became visible. With further decreases in the HRT below 4 h the growth increased and bacterial granules began to form and accumulated at the surface of the expanded CAC bed. After granulation had been initiated, further reductions in the HRT to between 2 and 1 h resulted in the rapid growth and expansion of the granular bed. The bioreactor was operated for a period of 32 days.

3.3.2 Bioreactor parameters monitoring

The bioreactor was operated for a period of 32 days at constant temperature of 65ºC with stepwise decrease in HRTs, where HRT= (bioreactor total volume)/ (nutrient influent rate): total bioreactor volume was approximately 7.5 L. The monitoring parameters were bioreactor temperature, pH, sucrose concentration, ammonium concentration, volatile fatty acids (VFA), biogas content and H_2 production during the course of experiment. The hydrogen production efficiency was evaluated using hydrogen gas content, hydrogen productivity (the ability converting COD into hydrogen, HP) and hydrogen production rate (the rate of hydrogen production from the reactor, HPR). All measurements represent the average of four to six replicates.

3.4 Analytical methods

3.4.1 Gas analysis

Gas chromatography was used to analyze % gas composition (H_2, CO_2 and CH_4). A Clarus 500 GC PerkinElmer equipped with thermal conductivity detector was used. The temperatures of injector, detector and column (PerkinElmer Elite Q Plot capillary column 30 m x 32 mm) were kept at 250 °C, 200 °C and 45°C, respectively. Argon was used as carrier gas at a flow rate of 2.0 ml min^{-1}. Sample gas injection volume was 20µl. The following formula (equation 3) was used for converting total bioreactor gas flux (L/h) to mmol H_2/h,

$$\frac{\Delta H_2}{\Delta t} = \frac{P\left[\left(\%H^{GC}\right)\frac{\Delta V}{\Delta t}\right]}{RT}. \qquad \text{.....Equation 3}$$

Where, $\Delta H_2/\Delta t$ = mmol H_2 /h; P = atmospheric pressure (85 kPa); $(\%H_2{}^{GC})$ = percentage hydrogen content from GC measurements; $\Delta V/\Delta t$ = L/h of total gas production from the gas meter measurements; R is the gas constant (8.314 J/ (K.mol)); T = 298.15 K.

GC calibration standards for measurement of hydrogen content

The steady-state gas stream produced by the bioreactor consisted of H_2 and CO_2. To determine the concentration of both H_2 and CO_2 in the total gas produced, the calibration standard curve was developed by injecting a three point standard calibration curve (one of our calibration gases comes with the gas composition: 45 % H_2, 2 % CO and the balance CO_2 or 100-45.13 -2.0 = 52.87 % CO_2). The plot graphs of area versus % H_2 and CO_2 for each injection were generated.

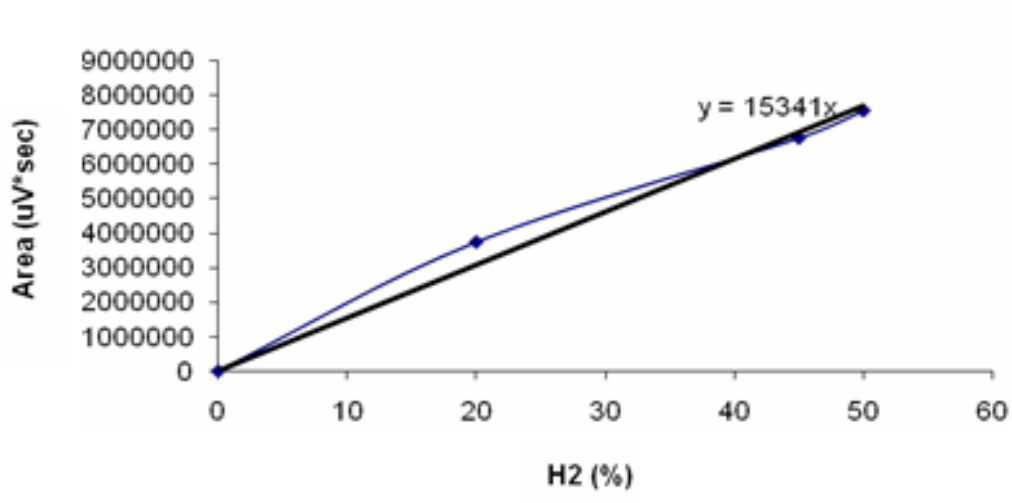

Figure 3.2: The plot graph of area versus H_2 % for each injection.

3.4.2 Volatile fatty acids analysis (VFAs)

Detection of VFAs (acetate, propionate, and butyrate) and solvent (ethanol), produced during fermentation in the bioreactor was performed by gas chromatography using the Varian 3300 FID GC equipped with a CP Wax 58 (FFAP) capillary column (25m x 0.53 mm). Before performing any liquid measurements, samples were subjected to filtration using a 0.22 µm membrane filters.

3.5 Determination of sucrose concentration

The concentration of sucrose in the reactor effluent and feed was measured colorimetrically using the sucrose-resorcinol method (Kerr et al., 1984). A solution of resorcinol reagent was prepared by dissolving 0.1 g resorcinol in 100 ml of 95% ethanol and 30% HCl was also made. A sucrose stock solution was prepared by dissolving 17 g of commercial sucrose into 1 L dH_2O. Thereafter, sucrose standard curves were then generated by mixing a known dilution of this standard (sucrose standard solution) with dH_2O to a total volume of 1 ml in 10 ml test tubes. For the sucrose colorimetric assay, each sucrose standard curve dilution (1 ml) was mixed with 0.75 ml of 30% HCl and 0.75 ml of the resorcinol reagent and then incubated in at 80°C for 8 min, after which 2 ml dH_2O was added to the sample. A spectrophotometer set at 520 nm was used for sucrose measurement against blank made with dH_2O water as

reference. Before performing colorimetric sucrose test, bioreactor effluent samples were subjected to a filtration using 0.22 μm membrane filters, then 1 ml of sample was used for sucrose determination according to above described method.

3.6 Determination of ammonium concentration

3.6.1 Assay solutions

Ammonia assay solutions were prepared according to phenol hypochlorite method described by (Solórzano, 1969; Russell, 1944). The oxidizing solution was prepared by diluting 25 ml of hypochlorite (commercial bleaching agent, Pick 'n Pay Brand, South Africa, labeled 3.5% hypochlorite) to 100 ml of sodium citrate solution (which was prepared by dissolving 100 g of trisodium citrate and 5 g NaOH in 500 ml dH_2O). The phenol solution, 10 % was made by dissolving10g phenol in 100 ml of 95% ethanol and 5% propanol. The sodium nitroprusside (was prepared by dissolving 1 g of sodium nitroprusside (Na_2-Fe (CN) 5NO.$2H_2O$) in 200 ml dH_2O.

3.6.2 Sample preparation and ammonia measurements

A stock solution of ammonia (NH_4Cl) was prepared by dissolving 0.5349 g of NH_4Cl (Merck) in dH_2O in a 1-liter volumetric flask. The ammonium standard curves were then generated by mixing a known dilution of this standard (ammonium stock solution) with dH_2O to a total volume of 5 ml in 10 ml test tubes. The following standard dilutions were made: 0.5 mM, 0.35 mM, 0.25 mM, 0.05 mM, 0.025 mM, 0.025 mM and 0.01 mM. The colorimetric ammonia determination on both bioreactor effluent samples and standard were done by the adding to the 5.0 mls the following reaction chemicals: 0.2 ml 10% phenol solution, 0.2 ml sodium nitroprusside and 0.5 ml oxidizing solution. After the addition of the reaction chemicals, the samples were left to stand for 1 h at room temperature and then the absorbance was measured at 540nm (BOECO S-20 Spectrophotometer, Germany), against a blank made with dH_2O as reference.

3.7 Total bioreactor bacterial biomass determination

The total biomass concentration in the reactor was determined gravimetrically. A 20 milliliters of sample suspended with bacterial cell was removed from the bioreactor and passed through 0.22 μm membrane filters. The residue collected on the filter was dried in an oven (3081U, labcon) at 65 ℃ set at rpm 86 for 48 hours. Thereafter the filter was weighed after dried to determine the mass of the biomass within the bioreactor.

The total bioreactor biomass was determined using the following formula,

$$\frac{\text{VOLUME OF SETTLED BED}}{\text{20 ml}} \quad X \quad DM$$

where DM is the settled granule dry mass per 20 ml.

3.8 Light dissecting microscope

In this study, light microscopy using a Dialux EE20 equipped with a digital camera was used to monitor the growth of biofilms and granules in the bioreactor. Granules diameter sizes were determined at every HRT in this study. Prior to measurements, the granules were removed from the bioreactor using a sterile plastic spatula (at every 2-4 days), and rinsed twice with distilled water to remove unattached bacterial cell and viewed under light dissecting microscope. In addition, the digital compact camera, C-7070 with wide zoom (Olympus imaging. Corp, Japan) was used to take image of the granules.

3.9 Scanning electron microscope (SEM)

SEM was performed using a method described by Lindsay and von Holy, (1996), with some modifications. A few granule samples were removed from the reactor (at every HRT), and the granules were rinsed twice with distilled water to remove unattached bacterial cells. The granules were then fixed in 3% (v/v) gluteraldehyde (Merck) for over overnight at room temperature. After fixing, the granules were sequentially dehydrated in a graded ethanol series (10, 20, 30, 40, 50, 60, 70, 80, 90 and 95 %), for 10 minutes each and stored in 100% (v/v) ethanol (Merck). The granule samples were then removed from ethanol and were subjected to critically point drying (HITACHI, HCP-2 Critical point Dreyer). The granules were mounted and coated with thin gold-palladium and allowed to air-dry, and observations were made with a JEOL, JSM-840 Scanning Electron Microscope operated at 20 kV (Lindsay and von Holy, 1996).In the addition, the microbial composition was also studied by standard gram stain technique.

3.10 Microbial community analysis by PCR-DGGE technique

3.10.1 DNA extraction

A 10 ml sample suspended with granule from the reactor was centrifuged at 10 000 x g for 5 minutes to collect bacterial cells. Genomic DNA was extracted from sample using the Zymo Research ZR Fungal/ Bacterial DNA kit ™, according to the manufacture's instructions (Inqaba Biotechnical Industries, South Africa). According to manufacture's instruction, Pellets were suspended in 1 ml ZR BashingBead™ Lysis tube (with 0.2 g of 0.1 mm glass bead) and vortexed at 14 000 x g maximum speed for 5 minutes, followed by centrifuged at 10 000 x g for 1 minute. Four hundred microliters of the upper aqueous phase was transferred into a Zymo-Spin IV^{TM} Spin Filter in a Collection Tube and centrifuged at 7 000x g for 1 minute. Then 1200 µl of Fungal/Bacterial DNA Binding Buffer was added to the subsequent filtrate where 800 µl of the mixture was transferred to the Zymo-Spin II^{TM} Column in a new collection tube followed by centrifugation at 10 000 for 1 minute, and a pre-wash DNA was done by adding 200 µl DNA Pre-Wash Buffer and followed by centrifugation at 10 000 x g for 1 minute. Afterwards 500 µl of Fungal/Bacterial DNA Wash Buffer was added to the Zymo-Spin II^{TM} Column in a new collection tube followed by centrifugation at 10 000 x g

for 1 minute. Finally 100µl of DNA Elution was added to elute the DNA in a clean 1.5 ml micro-centrifuge tube. No DNA concentration step was done, the purity of DNA was determined by measuring the absorbance at 260/280 nm.

3.10.2 Polymerase chain reaction (PCR) amplification

The 16 rDNA was amplified using the fermentas reverse primer UNIV 1392R and forward primer EUB 968F with GC clamp (Chang et al., 2006). The primer sets used for the amplification of 16S rDNA are listed in table 3.1. PCR was performed in 2 separate PCR tubes (Biorad) in a final volume of 50 µl. The first tube was composed of 25µl of 2X PCR Master mix (*Taq* DNA polymerase (recombinant) in reaction buffer, $MgCl_2$ and dNTPs 0.4 mM of each), 1 µl of each primer EUB968F with GC clamp and UNIV1392R, 1 µl genomic DNA and 22 µl DNase and RNase-free water (Fermentas, USA).

Table 3.1: Primers sequences used for 16S rDNA amplification in this study.

Name	Sequence	Bases	3' Mod	5' Mod
UNIV1392R	ACG GGC GGT GTG TRC	15	None	None
EUB968F	AAC GCG AAG AAC CTT AC	17	None	None
GC-EUB968F	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC	57	None	None

The second tube was composed of: 25µl of 2X PCR Master mix (*Taq* DNA polymerase (recombinant) in reaction buffer, $MgCl_2$ and dNTPs 0.4 mM of each), 1 µl of each primer EUB968F with GC clamp and UNIV1392R, and 23 µl DNase and RNase-free water (Fermentas, USA), and this tube was used as a negative control for this experiment since it contained all the reaction components except genomic DNA. This was done in order to test that no self amplification or DNA contamination occurred.

All the PCR tubes were briefly centrifuged at 10 000 rpm for 15 s to settle all the PCR reaction components. An automated thermal cycler (Applied Biosytems 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 (30 sec at 94 °C), annealing (45 min at 60 °C) and extension (1 min 30 sec at 72 °C), and a final extension at 72 °C for 7 min before storage at 4 °C.

3.10.3 Agarose gel electrophoresis

A 1 % (w/v) agarose gel was used to test and confirm the stability of the amplified DNA. To confirm the presence of amplified DNA, A 1 % (w/v) agarose gel was prepared by dissolving 1.5 g of Molecular Grade agarose (low EEO, Whitehead Scientific) into 150 ml of 1 X Tris-Borate EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). The solution was mixed by gently shaking in a 200 ml bottle, dissolved in a microwave oven for about 45 s and allowed to cool to about 45 °C. Two microlitres of ethidium bromide (10 mg ml^{-1}, Biorad) was added and mixed gently by shaking. The mixture was poured gently into a gel tray and allowed to solidify for about 20 min. A comb was inserted into the gel tray to form wells (lanes) into which the PCR products can be loaded. After solidifying, a comb was removed and the gel tray transferred into an electrophoretic tank (Biorad). A 1 X TBE buffer was added into the tank, such that it covered the gel. Two microlitres of 6 X (v/v) orange loading dye (Fermentas Life Sciences, South Africa) was placed on a sheet of parafilm and mixed thoroughly with 5 µl of 1 kb DNA ladder (0.1 µg $µl^{-1}$) (O`GeneRuler, Fermentas Life Sciences, South Africa) and also the mixture of orange loading dye with PCR products was made (5 µl of the PCR products). The molecular weight marker was loaded into the first lane and PCR products were loaded into the following lanes, according to standard loading technique.

3.10.4 Denaturing gradient gel electrophoresis (DGGE)

The diversity of the biohyhdrogen producing communities at each HRT was determined by using DGGE. DGGE was performed using a D-Code Universal Mutation Detection System (Bio-Rad Laboratories, California., USA). PCR product was directly applied to 8 %(w/v) polyacrylamide gel containing a 20– 60 % gradient of urea and formide. The 100 % denaturant was defined as 7.0M urea and 40% deionized formide based on the protocol of Muyzer et al., (1993). The electrophoresis was run in a 0.5X Tris-acetate-EDTA (TAE) buffer solution at a constant voltage of 130V and 65 ºC for 5 hours. After electrophoresis, the gel was stained in 250 ml of 0.5X TAE buffer containing 2.5 µl of a 10 $mg.ml^{-1}$ ethidium bromide. Subsequently, the stained gel was distained for another 15 minutes in 250 ml of 0.5X TAE buffer this time without the ethidium bromide. The gel was visualized in the Gel Doc. The bands in the gel were carefully excised with a razor blade under UV illumination, and then placed in 100 µl of TE buffer. DNA was extracted from the gel piece by overnight incubation at 4ºC, and then 1 µl supernatant was used as template DNA in the re-amplication by PCR using UNIV 1392R and EUB968F primer without a GC-clump this time. The resulting PCR product was sent for sequencing.

3.10.5 DNA sequencing, subtyping and phylogenetic analyses.

Sequencing was performed by Stellenbosch University Sequencing Unit (South Africa) using an ABI3130xl Genetic analyzer. The DNA sequences were edited using FinchTV Version 1.4.0 software (www.geospiza.com) and manual adjustments were made where necessary.

For subtyping, edited DNA sequences were submitted to the BLAST program in the GenBank database of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/GenBank/), using the Basic Local Alignment Search Tool BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The DNA sequences and GenBank reference sequences were multiply aligned using the multiple sequence alignment tool DNAMAN Version 4.0 software (Lynnon Biosoft, Department of Microbiology, University of Cape Town), and unrooted phylogenetic trees were constructed using the neighbor-joining method (DNAMAN Version 4.0) with a bootstrapping stringency of 1000, so as to reveal the

phylogenetic relationship and degree of relatedness between DNA sequences and GenBank reference sequences.

CHAPTER FOUR

RESULTS

4.1 Bioreactor design and operation strategy

In this study, it was necessary to implement a number of modifications in the bioreactor design and operation strategy, from our old anaerobic fluidized bed bioreactor (AFBR) prototype in order to improve both the Hydrogen productivities (HPs) and hydrogen yields (HYs), (The Evolution of Wits bioreactor prototype, shown in figure 4.1). Firstly, the total volume of the original bioreactor system had to be reduced substantially from 10L to either 7.5L or 5L. The original bioreactor system had a total volume of 10 L, with settling column place above the 5 L bioreactor contributed to increase in the total system volume. The purpose of the settling column was incorporated in the bioreactor to function as the granule settling tank, as shown in figure 4.1. However, observation were made that bioreactor with settling column always had HY less than 2 mol H_2/mol of glucose. The removal of settling column was suggested, and removal of the settling column always had an effect on HY. Secondly, following the reduction in the volume of the bioreactor system, the HPs and HYs were improved by bioreactor operational strategy. This was done by operating the bioreactor at increasing effluent recycle rate from 1.3 L/min to 3.5 L/ min, the bioreactor for most of the duration of the experiment the effluent recycle rate was maintained at 3.5 L/min with simultaneous increases in the influent dilution rate (or reduction in HRT). Thirdly, the gas disengager was designed to assist in the reduction of the hydrogen concentration trapped in the liquid bulk phase. This was accomplished by facilitating stripping of the H_2 from the liquid phase within the gas disengager to the vapour phase, which is being continuously exhausted from the gas disengager. The above mentioned modifications, improved the ability of the reactor to reduce the hydrogen partial pressure within the reactor.

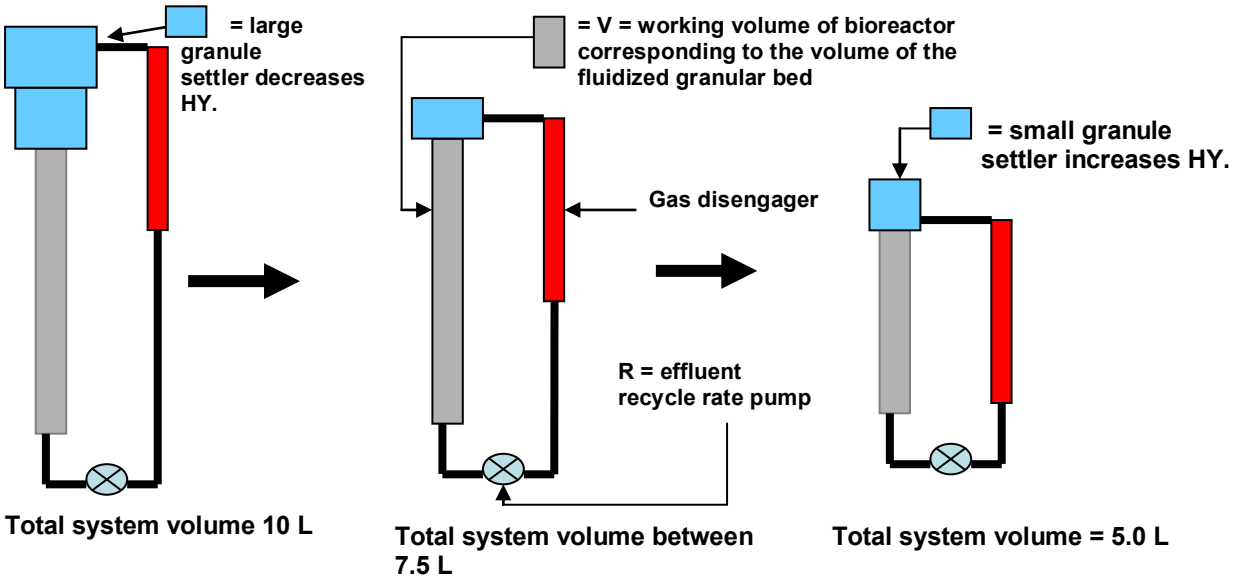

Figure 4.1: Evolution of the anaerobic fluidized bed bioreactor (Wits Bioreactor Prototype, AFBR).

4.2 Formation of bacterial granules

After the post-inoculation 48 h batch-effluent recycle acclimatization period the bioreactor operation was switched to continuous degassed effluent recycle mode with an initial influent rate of 0.75 L/h which corresponded to an HRT of 10 h, where HRT = (7.5 L system volume)/(influent rate), and an effluent recycle rate of 1.3 L/min. The HRT was kept at 10h for 2 days (48 hours), followed by HRT 8.3 h for 3 days after which time biofilm growth became visible on the top of the cylindrical activated carbon (CAC) bed, as shown in figure 4.2. Bacterial granule initiation, growth and development were promoted by the sequential stepwise decrease in HRTs and sequential stepwise increase in the effluent recycle rate at 3 day intervals. Following the initiation biofilm development on the CAC bed the influent rate (L/h) and effluent recycle rate (L/min) was increased every 3rd day as follows: 0.9 L/h (HRT = 8.33h) and 1.3 L/min, 1.8 L/h (HRT = 4.17h) and 2.0 L/min, 2.7 L/h (HRT = 2.7 h) and 2.6 L/min, 3.6 L/h (HRT = 2.08h) and 3.2 L/min, 4.5 L/h (HRT = 1.67h) and 3.5 L/min. At a HRT of 4.7 h and with an increase in the degassed effluent recycle rate to 2.0 L/min bacterial flocs started to form in the bioreactor bed. Further reduction of the HRT to 2.78 h and with an increase in the degassed effluent recycle rate to 2.6 L/min granules began to form on top of the expanded CAC bed grew to a settled bed height of 9cm, figure 4.2. Following the establishment of a steadily growing granular bed the HRT was further reduced to 2.08 h and

the effluent recycle rate was increased to 3.2 L/min. Under these conditions the granules grew to a settled bed height of 12 cm. With a further reduction of the HRT to 1.67 h and an increase in the degassed effluent recycle rate to 3.5 L/min the granules grew to a settled bed height of 13.5 cm. At this stage any further increase in the influent feeding rate resulted in granule washout from the reactor. During days 20 to 24, in response to an influent rate of 4.5 L/h and effluent recycle rates of 3.5 L/min the fluidized granule bed occupied the full bioreactor working volume of 5.027 L, with the fluidized bacterial dry mass (DM) density of 35.1 g DM/L. At this stage of granule development the granules became non-friable and did not disintegrate in response to the abrasive shear forces generated by effluent recycling rates of 3.5 L/min, some of the granule images are shown in figure 4.3. It was found that the growth of the granular bed was promoted by steadily increasing the effluent recycle rate following each reduction in HRT. In addition, formation of biofilms and/or granules in this study was accomplished in a very short period of 5 to 9 days, after the sludge seed was subjected to an acid incubation for 24 h by shifting the culture pH from 5.5 to 2.0. The seed sludge was originally dark in color and gradually whitened, and became creamy white by day 4. The dark color in the seed sludge was due to the presence of sulfide produced by the sulfate-reducing bacteria and low sulfate concentration in the synthetic wastewater, as the reactor was increasingly feed the residual of sulfide precipitates and sulfate-reducing bacteria were gradually washed out from the bioreactor.

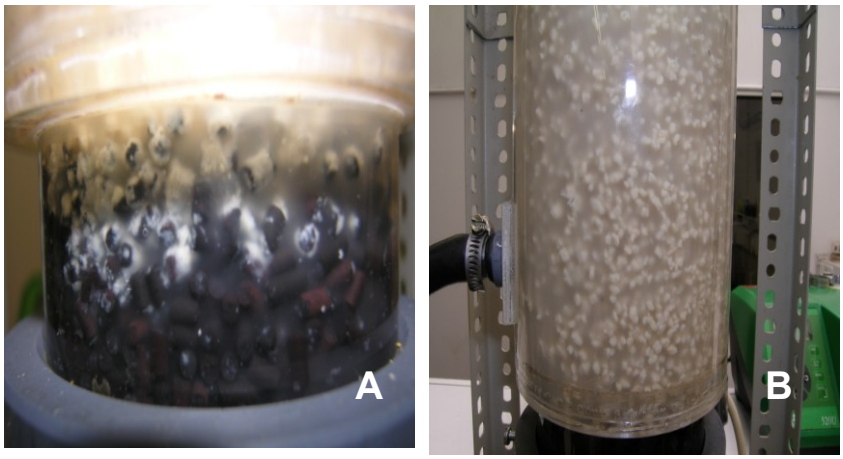

Figure 4.2: (A) Image of the AFBR column with activated carbon carrier (CAC) coated with bacterial flocs (biofilms), (B) Shows the full bed bed granulated AFBR reactor during fermentative biohyhdrogen production.

4.2.1 Physical characteristics of the granules

Table 4.1 summerizes the physical characteristics of H_2 producing granules in this study. Several granules shapes were observed, these includes: spherical, elliptical, irregular shapes, nodulated granules. The average size of granules diameter varied from 1.5 to 2.5 mm (image of the granules taken by using light dissecting microscope are shown in figure 4.4). These granules had settling velocities which ranged from 90 cm/min to 246 cm/min (see table 4.1). Based on the granules increased size diameters, definitely bacterial biomass retention within the bioreactor bed was facilitated, in the presence of the uplifting forces generated by recycle effluent flow velocities.

Table 4.1: Physical properties of hydrogen producing thermophilic granules

Days	Settled bed height (cm)	Granule diameter (mm)	Granule settling rate (cm/min)	Granule morphology	Granule stability
6	Bacterial flocs				
9	4				
12	10	0.7			Wash-out
15	12	0.6	1.5		Wash-out
18	12.5	0.81	90	Irregular and nodulated	unstable
21	13	2.2	196	round, nodulated	stable
24	13.8	2.5	246	round	non-friable

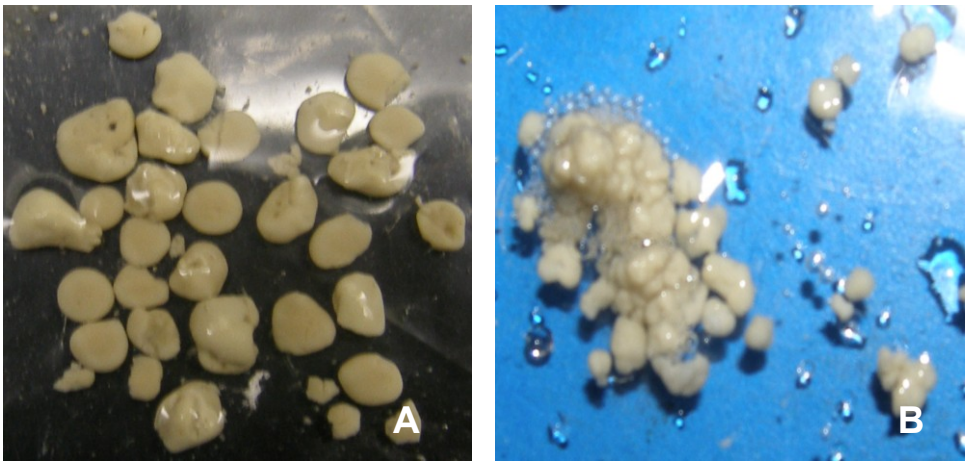

Figure 4.3: Pictures of H_2-producing granules: A) H_2-producing granules at day 11; B) H_2-producing granules at day 22. Image of the granules were taken using a HD 620 samsung camera model.

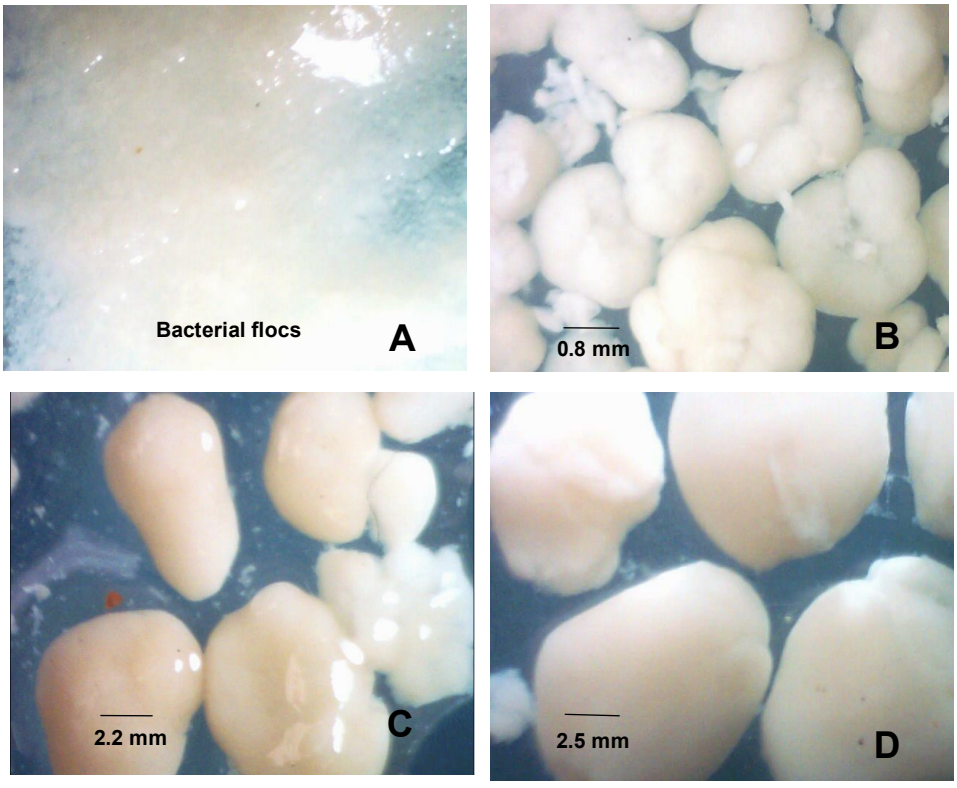

Figure 4.4: Bacterial biomass studies with respect granules formation and their development stages into matured granules during anaerobic biohyhdrogen, using a light dissecting microscope A) H_2 producing bacterial flocs became visible at the bottom of the bioreactor after 6 days of operation. These bacterial flocs grew rapidly, and after day 9 granules were formed; B) spherical granules; C) elliptical granules; and D) matured granules with diameter of over 2.5 mm were formed, at this stage the granule growth rate became slower, indicating the mature and stable granule formation.

4.2.2 Morphology of the Granules

A close examination of the bioreactor sample by gram staining revealed that bacterial population in the reactor consisted of rod shaped bacteria, figure 4.5. However, a much detailed study of morphology of granules was studied by scanning electron microscope (SEM). Figure 4.6 and 4.7 show SEM images of the typical granules between day 12 and 22. SEM images revealed that microbial community in the granule consisted predominantly of rod-shape bacteria. It was observed that, on the surface of bacterial granules there exists a porous inner structure or presence of cavities. Such structure is likely to facilitate the passage of nutrients and substrate as well as the release of metabolic products such as biogas (H_2) from the granules.

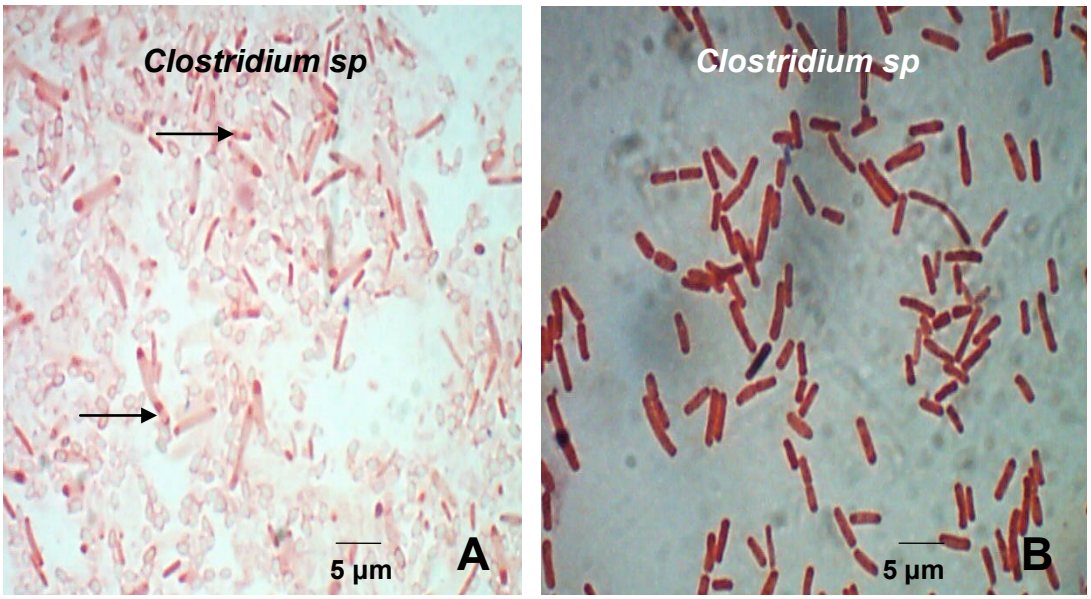

Figure 4.5: Gram stain images, sample culture from the AFBR reactor: A) Sporulating rod shaped cells indicated with an arrow; and B) rod-shaped cells.

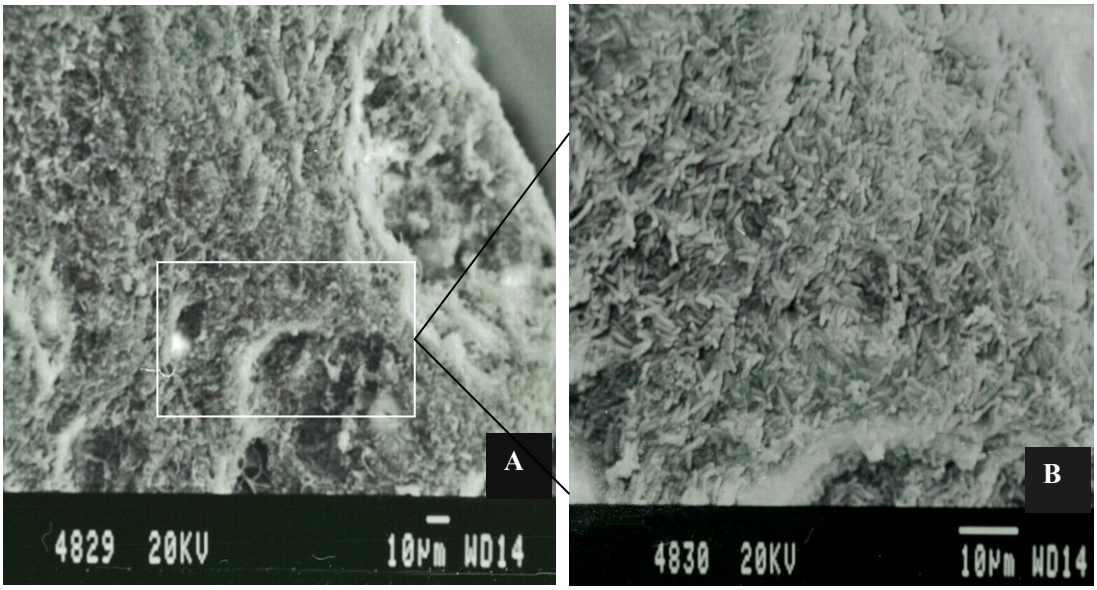

Figure 4.6: SEM images of (A) overview of the granules surface and (B) Image of sectioned granule showing the porous structure of the surface of the hydrogen-producing bioparticles.

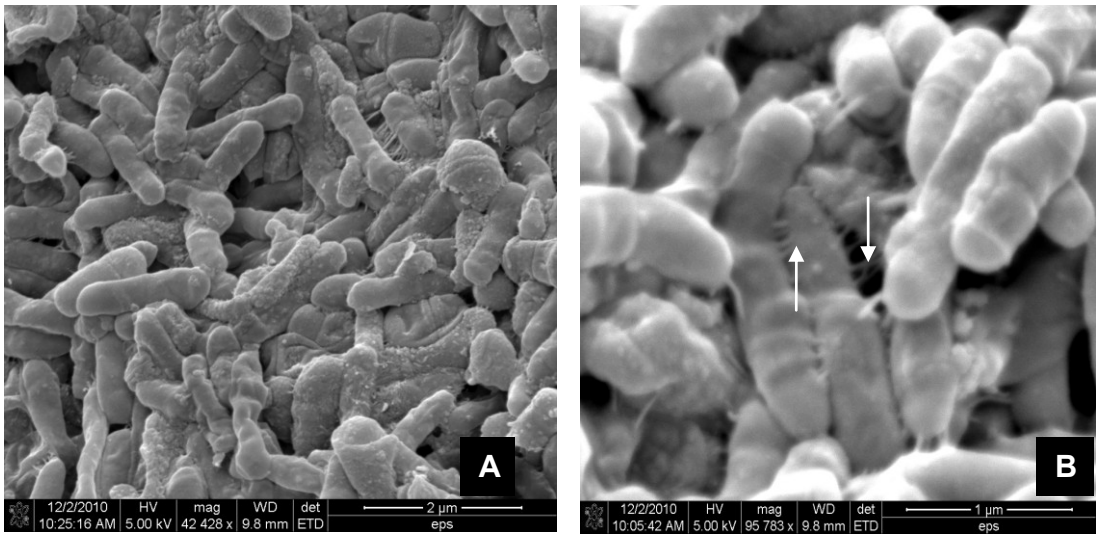

Figure 4.7: SEM photographs showing bacterial morphology on the surface of the granules: A) spore-forming rod shape bacteria; and B) the arrow show extracellular polymers for bacterial attachment.

4.3 Thermophilic bioreactor performance with respect to: Hydrogen production rate (HPR), hydrogen productivity (HP), Hydrogen content (%), Hydrogen yield (HY), Sucrose conversion rate, distribution of soluble metabolites, during 27 days of operation.

Hydrogen production was detected starting from day 3 (at HRT 10 h), as the HRT was gradually decreased from 8.33 h to 4.16 h the hydrogen production rate (figure 4.8) and hydrogen yield (figure 4.9) increased from 0.9 L H_2/h and 0.66 mol H_2 /mol glucose to 3.2 L H_2/h and 1.12 mol H_2/mol glucose, respectively. The effect of effluent recycle rate on both HPR and hydrogen content (%) is shown in figure 4.10, as the effluent recycle rate was increased from 1.5 L/min to 3.5 L/min and a decrease in the HRT to 1.67 h resulted in an HPR of 32.7 L H_2/h, while hydrogen content of 54% was found at 1.67 h HRT. Hydrogen (H_2) and carbon dioxide (CO_2) were produced as gaseous products, and no methane (CH_4) was detected during the course of the experiments, suggesting that the acid and heat pretreatment methods were effective at inhibiting the activity of methanogenic bacteria in the anaerobic sludge. In this study a maximum hydrogen productivity of 1100.6 mmol H_2/h was achieved (figure 4.11). The experimental results showed that both HPR and hydrogen yield increased significantly with the shortened HRT, giving the maximum at the shortest HRT of 1.67 h of 32.7 L H_2 / h and 3.905 mol H_2 / mol, respectively (both plots are shown in figure 4.8 and 4.9).
However, the sucrose conversion rate decreased apparently, from 98 % at 10h to 60% at 1.67 h HRT (Figure 4.12). Regardless of decrease in sucrose conversion efficiency the production of hydrogen increased and also hydrogen composition increased with the reduction in HRTs.

The changes of the hydrogen production at different pH values from 3.5 to 7.2 is shown in figure 4.13. During the course of the experiments the hydrogen production increased with increasing pH, with the maximum hydrogen production rate of 32.7 L H_2/h attained at pH 7.02 (see figure 4.13). We suspect that the sharp increase in the effluent pH was as a result of low concentration of the acetate, propionate and butyrate in the bioreactor effluent. The effluent was mainly composed of acetate, propionate and butyrate, the results showed that the concentration of acetate, propionate and butyrate

declined from 16.01 mM, 6.43 mM and 6.42 mM to 5.19 mM, 2.05 mM and 1.31 mM, respectively (see table 4.2).

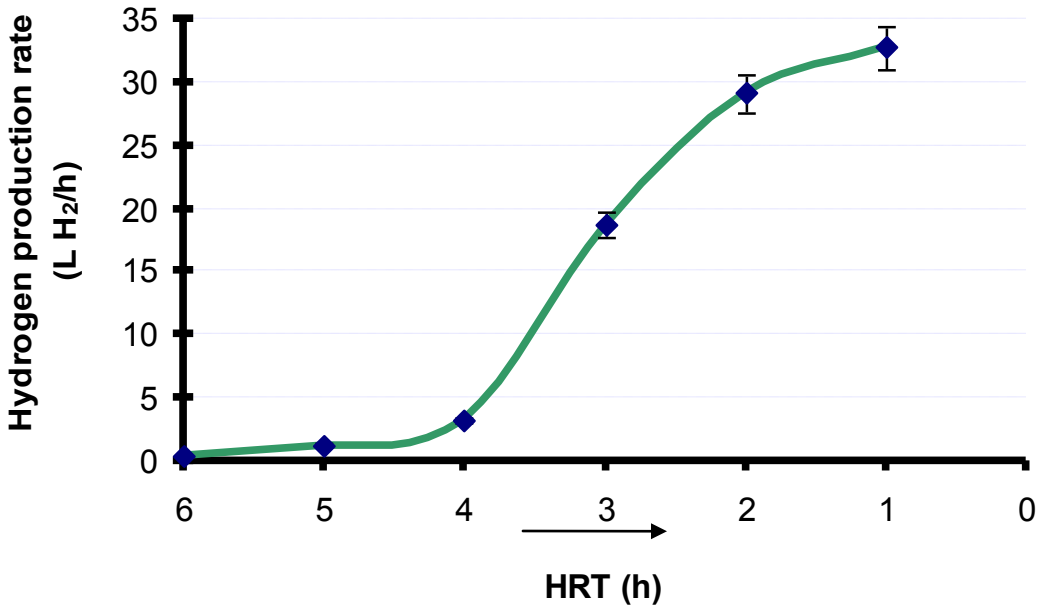

Figure 4.8: The effect of hydraulic retention time on hydrogen production rate at a constant effluent recycle rate of 3.5 L/min. To read this graph, the arrow represents the direction of HRT change during the experiments.

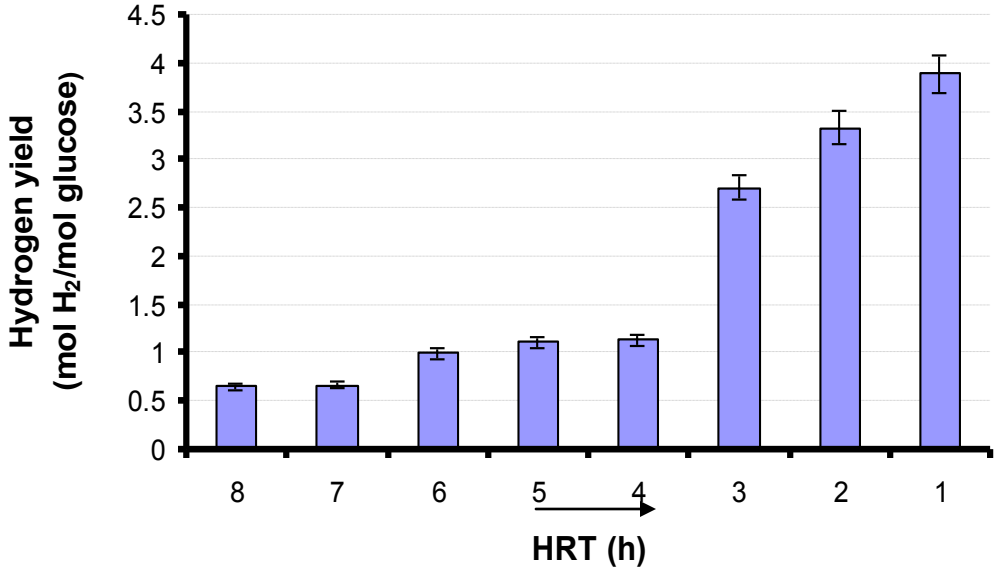

Figure 4.9: The effect of hydraulic retention time on the hydrogen yield at a constant effluent recycle rate of 3.5 L/min. To read this graph, the arrow represents the direction of HRT change during the experiments.

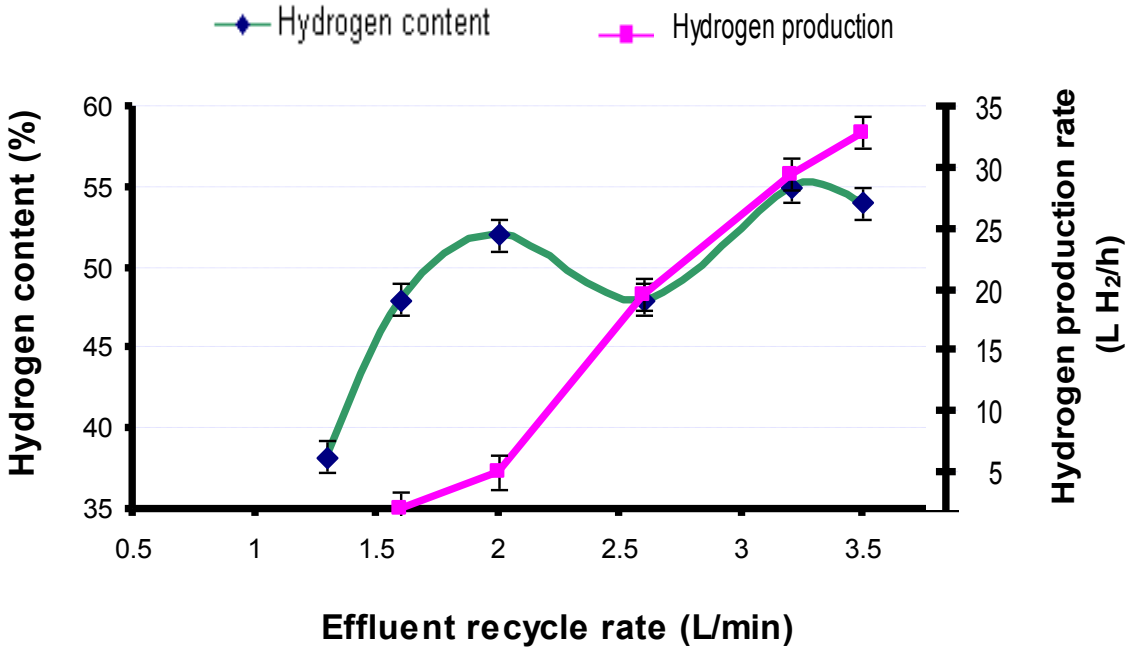

Figure 4.10: Effect of effluent recycle rate on hydrogen content and hydrogen production rate, the influent rate (L/h) and effluent recycle rate (L/min) was increased as follows: 0.9 L/h (HRT = 8.33h) and 1.3 L/min, 1.8 L/h (HRT = 4.17h) and 2.0 L/min, 2.7 L/h (HRT = 2.7 h) and 2.6 L/min, 3.6 L/h (HRT = 2.08h) and 3.2 L/min, 4.5 L/h (HRT = 1.67h) and 3.5 L/min.

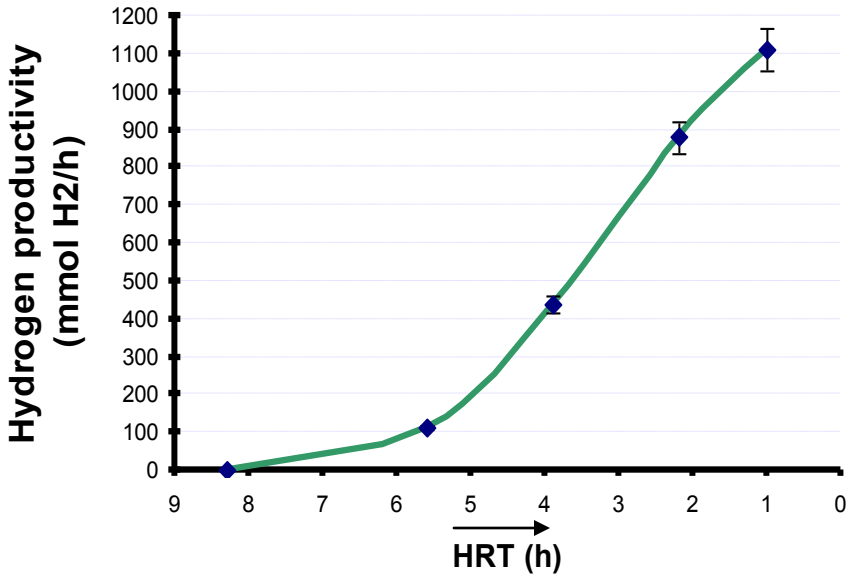

Figure 4. 11: The effect of HRT on the hydrogen productivity at a constant effluent recycle rate of 3.5 L/min. To read this graph, the arrow represents the direction of HRT change during the experiments.

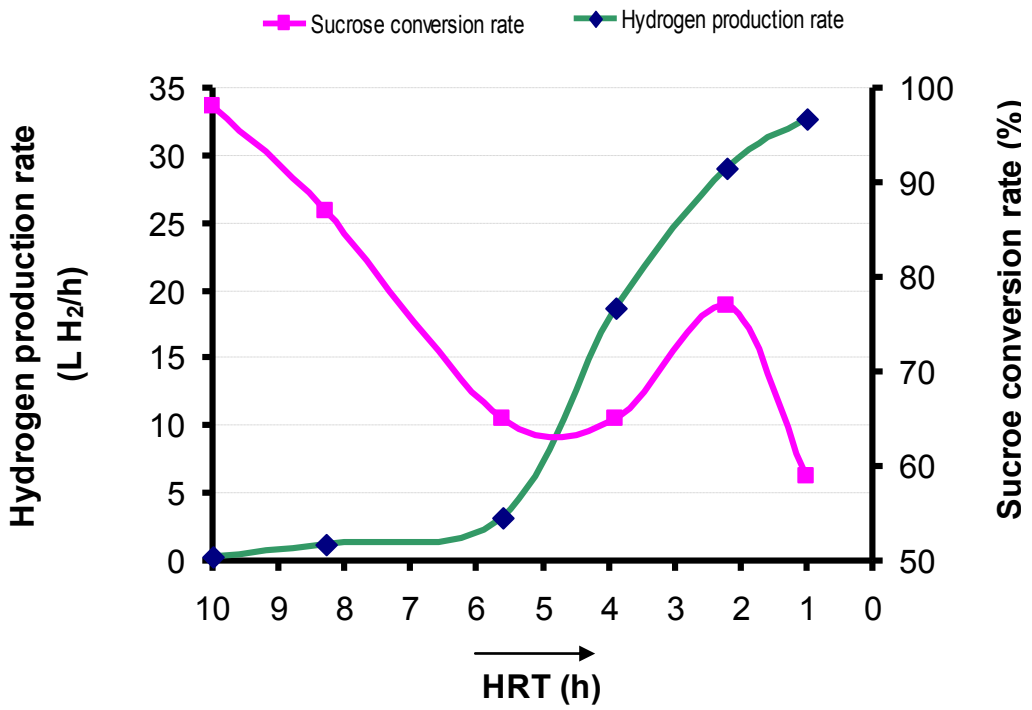

Figure 4.12: the effect of HRT on hydrogen production rate and substrate conversion at a constant effluent recycle rate of 3.5 L/min in the granule thermophilic reactor. To read this graph, the arrow represents the direction of HRT change during the experiments.

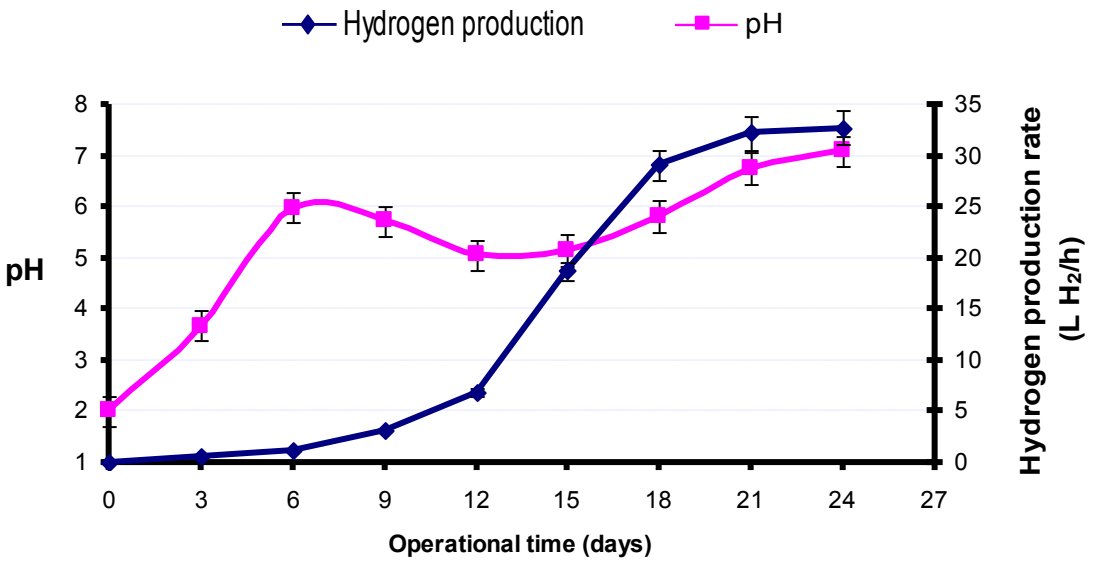

Figure 4.13: Long-term stability of the AFBR performance; time-coarse profile of hydrogen production rate and pH in the thermophilic AFBR operated at 65ºC during 27 days of operation. Following the initiation biofilm development on the CAC bed the influent rate (L/h) and effluent recycle rate (L/min) was increased every 3rd day as follows: 0.9 L/h (HRT = 8.33h) and 1.3 L/min, 1.8 L/h (HRT = 4.17h) and 2.0 L/min, 2.7 L/h (HRT = 2.7 h) and 2.6 L/min, 3.6 L/h (HRT = 2.08h) and 3.2 L/min, 4.5 L/h (HRT = 1.67h) and 3.5 L/min.

Table 4.2: Distribution of the soluble metabolites in the thermophilic AFB reactor

HRT [h]	Sucrose [mM]	Acetate [mM]	Propionate [mM]	Butyrate [mM]	ethanol [mM]
10	8.18	16.01	6.43	6.42	11.88
6.11	22.64	6.58	5.90	2.67	3.11
5.9	23.76	5.44	5.37	3.14	2.95
4	27.35	2.98	1.95	0.89	1.74
3	26.06	3.10	2.14	1.05	1.88
2.98	26.30	5.83	4.01	2.17	2.02
1.5	31.55	5.19	2.05	1.31	1.46

4.4 Microbial composition analysis

In order to find out the major bacterial populations of the microflora that contribute to hydrogen production in the bioreactor, total DNA was isolated from the bioreactor granules at different HRTs, and subjected to eubacterial 16S rDNA-targeted PCR-DGGE analysis by using EUB 968F with a GC-clamp and UNIV 1392R as the primers. It was found that PCR amplification produced a fragment of about 450 bp that was amplified, see figure 4.14.

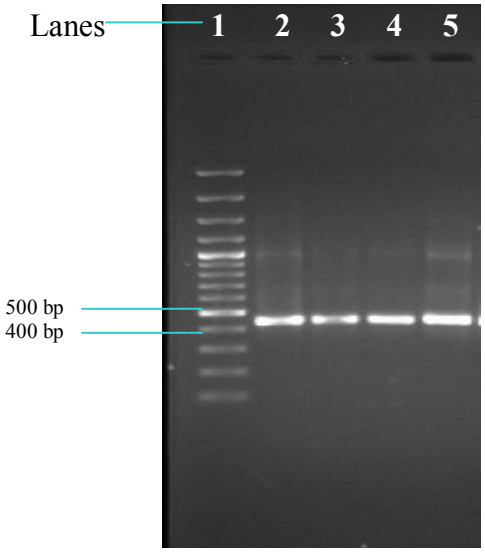

Figure 4.14: PCR-amplified genomic DNA of biohydrogen producing microorganisms at different HRT's (L1= DNA ladder, L2 and L3 correspond to HRT 5.6 h; L4 and L5 correspond to HRT 2.08 h). The GeneRuler^{TM} 1kb DNA Ladder was used on 1% agarose gel to determine the size of the isolated DNA fragments (450bp).

For a 65°C bioreactor operation the number for 16S rDNA PCR amplicons bands decreased from 6 to and 4 as the HRT was decreased from 10 h to 2.08 h. This suggests that with time as the HRT was decreased the consortium in the thermophilic granules was reduced to between 3 to 4 bacterial species (see figure 4.15).

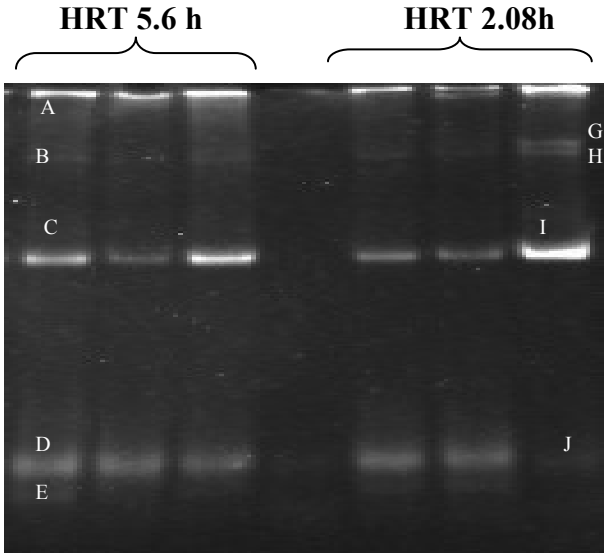

Figure 4.15: DGGE analysis of the partial bacterial 16S rDNA genes amplified from AFBR reactor at HRT 5.6h and 2.08 h. The PCR products were loaded onto 6% polyacrylamide gel in 1X TAE buffer (20mM Tris-acetate, 10 mM sodium acetate, 0.5 mM EDTA, pH 7.4) with a denaturing gradient (Urea-formamide) that ranged from 20-60%. The excised and sequenced bands correspond to the following bands: A: isolate FO1_PCM1; C: isolate A03_PCM3; D: isolate GO6_LP6; I: isolate BO6_co 1. Some of the bacterial bands were unsuccessfully identified (e.g. B, E, G, H and J). Excised DGGE band is indicated by alphabet (A,B,C,D,E,F,G,H,I,J)

The phylogenic tree illustrated in Figure 4.16, shows that the 6S rDNA sequences of the bioreactor samples were closely affiliated with the clostridia (C*lostridium thermopalmarium*, *Clostridium thermobutyricum*, and *Clostridium botulinum* strain, and *Clostridium butyricum*, *Clostridium pasteurianum*). Other species such as *Klepsiella species* were also found in this study. All members of the clostridia are capable of producing hydrogen from a wide variety of substrates including complex carbohydrates.

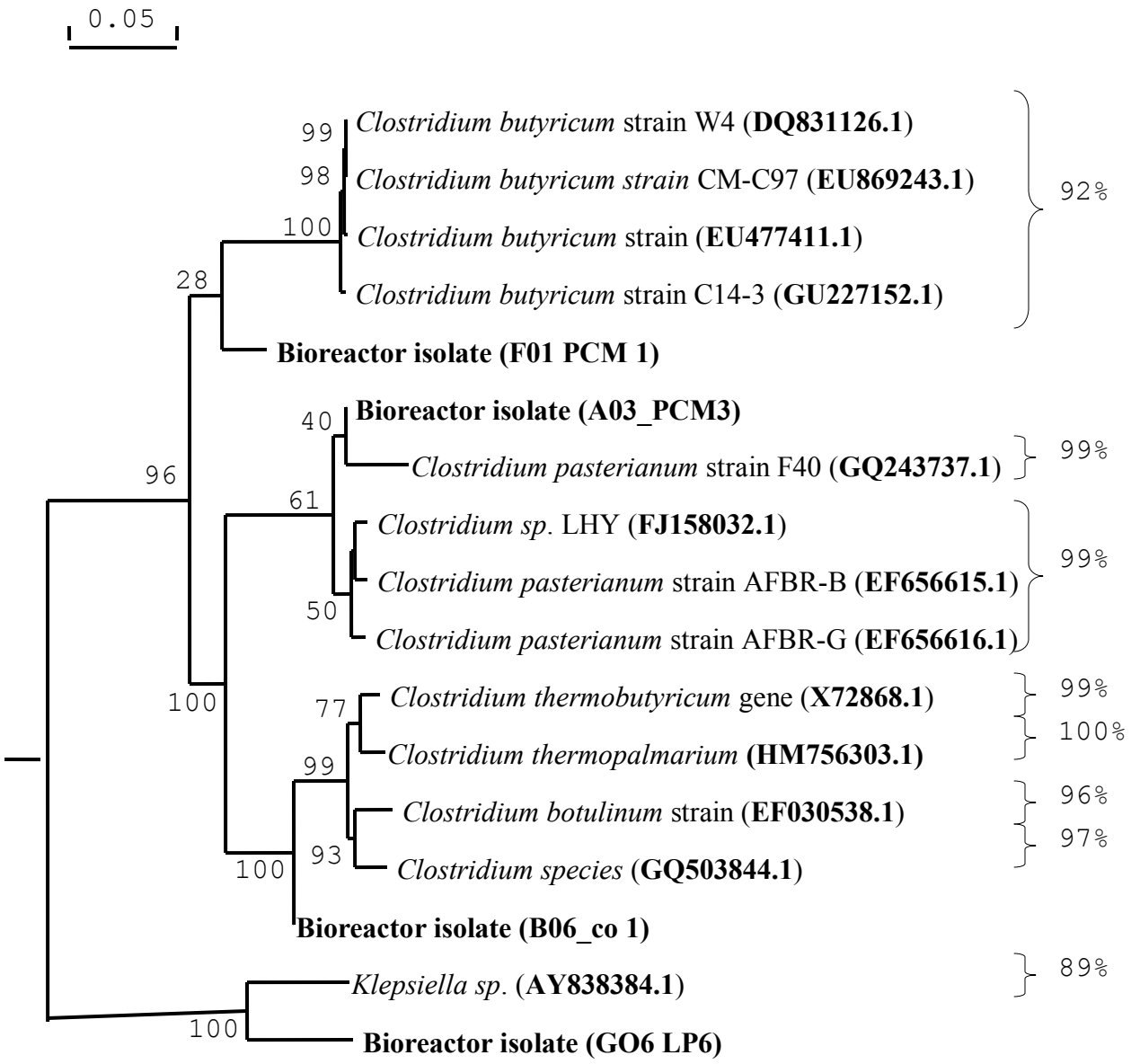

Figure 4.16: A 16S rDNA phylogenetic tree and their close relationship of sequences of the excised bands of the 16S rDNA genes amplified from AFBR reactor. The tree is based on DNAMAN distance was constructed using neighbor-joining algorithm with 1000 bootstrapping. The scale bar represence 0.05 nucleotide divergence. Nucleotide sequence accession numbers or strain names are indicated in brackets and also similarity (%) is indicated.

CHAPTER FIVE

DISCUSSION

5.1 Bioreactor Design and operational strategy

Recent advancement in H_2 fermentative process involves improving both H_2 productivity (HP) and hydrogen yield (HY) through bioreactor design. For industrial fermentative H_2 production, specialized bioreactor configurations with more robust, reliable performance that are stable for long periods of time (months) and resistant to short-term fluctuations in operational parameters are considered as ideal bioreactors. In this study, several modifications and operational strategies on the bioreactor were made in order to improve hydrogen production and removal from the sites of H_2 production. These modifications made it possible to achieve high hydrogen yield. Importantly, the newly designed bioreactor offered several advantages such as: high ability to retaining microbial biomass, efficient operation at low HRTs. Indeed, many studies recently have shown that high hydrogen production rates can be achieved through improved bioreactor configurations (Hawkes et al., 2007; Bartacek, 2007; Kim et al., 2005). Furthermore, this study satisfied that high HP and HY could be achieved through improved bioreactor design.

5.2 Bacterial granulation and physical characteristics of granules

Granulation is an efficient means of bacterial biomass retention in dark fermentation bioreactors, and thus enable high organic loading and H_2 productions (Lee et al., 2004a, b; Wang and Chang, 2008; Wu et al., 2006; Zhang et al., 2007).In this study, carrier induced thermophilic bacterial granulation has proven to be helpful in enhancing H_2 yield and providing stability to the process. The granules were formed within a period of 5 to 9 days, and during days 20 to 24, in response to an influent rate of 4.5 L/h and effluent recycle rates of 3.5 L/min the fluidized granule bed occupied the full bioreactor working volume of 5.027 L, giving a fluidized bacterial dry mass (DM) density of 35.1 g/L. Volumetric hydrogen productivity is directly proportional to the bacterial biomass density. Recent advances in the capacity to initiate the induction and, growth and development of anaerobic bacterial granules has made it possible to achieve the bacterial dry mass necessary for the achievement of HPs greater that 120 mol $H_2/(cm^3.h)$.

Bacterial dry biomass densities between 21.5 and 37 g/L are necessary for obtaining viable HPs (Lee et al., 2006; Zhang et al., 2007b, 2008b).

The granules formed in this study were characterized in terms of their physical and microbial morphology using scanning electron microscope and light dissecting microscope. The size of the most hydrogen producing granules in this study was in range of 0.7 - 2.5 mm (table 4.1). The granules had good settling ability and density which facilitated the bioreactor to be operated at a very low HRT of 1.67 h with minimum bacterial biomass washout in the AFBR. Their settling velocities improved as the HRT was reduce from 4h to 1.67 h, increased from 90 cm/min to 246 cm/min, respectively. This was evidence that induction of microbial granulation by short HRT is considered to be related to the hydrodynamic (and organic loading related) selective pressures (Lee et al., 2006). Because of high microbial cell retention in the reactor, it was evidence that formation of granules resulted to improved hydrogen production efficiencies.

In this study granules with high tensile strength were found to be necessary for the operation of the bioreactor presented in Figure 3.1. Without the granules possessing the tensile strength sufficient to withstand the corrosive action resulting from the exposure to the high forces, generated by the combine effect of high influent rates and with linear flow velocities between 5 and 100 cm/min and the high degassed effluent recycle rates with linear flow velocities between 100 and 1000 cm/min of effluent, it will not be possible to operate a thermophilic fluidized bed bacterial granular bed bioreactors under these operational conditions. Clearly, granulation played an important role in maintaining the stability of the bioreactor, such as enhancing biomass retention and generation of micro-environment that favors interspecies syntrophic interaction among bacteria involved in metabolism.

5.3 Thermophilic biohyhdrogen process performance of AFBR

The realization of hydrogen as a clean fuel for the future has triggered research around the world to look for novel methods for its production from renewable resources. In the past two decades, many studies investigating the efficiency of dark anaerobic biohyhdrogen production have been conducted under mesophilic temperatures. In spite of large number of studies conducted so far, H_2 yield have been quite low and stagnant. Recently, thermophilic fermentations are gaining increasing attension around the world, due to high hydrogen yields associated with them (Hallenbeck, 2005). Using (extreme) thermophiles, 1 mol of glucose can be converted to 4 mol of H_2 and 2 mol of acetic acid as the main product (Schröder et al., 1994, van Ooteghen et al., 2004; Zeida and van Niel, 2009), which is considered as the maximum theoretical yield achievable.

In this study, the application of influent rate of 4.5 L/h (HRT 1.67 h) and effluent recycle rate of 3.5 L/min resulted to the attainment of maximum hydrogen production rate and hydrogen yield of 32.7 L H_2/h and 3.91 mol H_2 / mol glucose, respectively. The reported hydrogen yield value in this study is very high and close to theoretical yield, this yield was achieved because of the better thermodynamic conditions in our thermophic bioreactor (such as high substrate loading rates, low hydrogen partial pressure and high bacterial biomass densities). Comparable, similar findings were reported by Zeidan and van Niel, (2010) , who reported that in a thermophilic fermentation with *Caldicellulosirupor owensensis* an HY of 4.0 mol H_2 / mol has been achieved (Zeidan and van Niel, 2010). Table 5.1, shows a comparison of hydrogen production rates and yields achieved in this study and those reported in literature. The results of this study indicate that high hydrogen production rate and yield can be simultaneously achieved in the anaerobic fluidized bed bioreactor. Lets look at the below reaction 12.

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2 \quad \Delta G^{o'} = -206.1 \text{ kJ/mol}$$

(Reaction 12)

Given the strongly negative $\Delta G^{o'}$ for the above reaction, it seems possible that of the 24 electrone equivalents (e^- eq) of glucose, 8 e^- eq should end up in H_2 with the remaining 16

e^- going to acetate. Because of internal bioreactor thermodynamic constrains dark fermentation hydrogen yields are usually below 4 mol H_2/mol glucose (Rittmann, 2008). Theoretically acetate could be further oxidized under anaerobic conditions to yield 4 H_2 and 2CO_2 in the absence of *methanogens* if the partial pressure is reduced.

It appears that for the anaerobic oxidation of glucose to hydrogen and acetate (reaction 12) the decrease in the ΔG^o from -206.1 kJ/mol at 25 ^oC to -223.7 kJ/mol at 60 ^oC was insufficient for overcoming the thermodynamic barrier necessary to achieve HY of 3.0 mol H_2 / mol glucose in a high rate thermophilic granular bed bioreactor (O-Thong et al 2008) with an HP of 152 mmol H_2/(L.h). The above $\Delta G^{o'}$ at 60 ^oC was based on a calculated enthalpy of 61.6 kJ/mol for the overall reaction and on an estimated entropy of 513.5 J/(mol.K) for H^+ ion production under cellular ionic and pH conditions, the latter value is consistent with reaction in equation 8 being an entropic driven process. Whether or not a practical viable anaerobic single or multi-stage bioprocess could be engineered, possibly with the application of external work in the form or another, that would remove the potential energy barriers preventing the complete oxidation of glucose to 12 H_2, remain an interesting, but controversial consideration (Hallenbeck, 2009; Hallenbeck and Gosh, 2009). In this study, we succeeded to overcome the thermodynamic constraints preventing the simultaneous attainment of both high HPs and high HYs by combination of external parameters such as thermophilic temperature, low HRTs and high recycles of de-gassed effluent.

Table 5.1: A comparison of hydrogen production rates and yields achieved in this study and those reported in literature.

Bacteria	Bioreactor System	HRT/ D*	Sub	^{o}C	pH	$\%H_2$	HP	HY	Ref
Sewage sludge	CSTR/AFBR	0.25 h	Glucose	37	5.5	38-48%	311	1.7	Zhang et al 2008
Thermobrachium celere/ thermoanaerobacterium aetearoense	CSTR	3 h	Glucose	58	-	-	45.8	1.54	Koskinen et al 2008
Enterobactar cloacae DM 11	Packed bed	1.08	glucose	-	-	36.8	75.6	2.04	Kumar and Das 2001
Thermophilic mixed cultures	TBR	4	Glucose	60	5.5	57-60	47.7	1.11	Oh et al., 2004
Mesophilic mixed cultrures	CIGSB	0.5 h	Sucrose	35	5.5	35.1	227	3.03	Lee et al 2004
Mixed culture sewage sludge	CSTR	6	Glucose	36	5.5			2.1	Fang et al., 2002
Mesophilic mixed cultures	CIGSB	0.5	sucrose			39.3	327	-	Wu et al., 2005
Sewage sludge	AFBR	1 h	Sucrose	65	7.09	55%	212.5	3.91	This study

Bioreactor system: CSTR, AFBR etc; HRT = hydraulic retention time; Sub = substrate; HP = hydrogen productivity mmol H_2 /(L.h); HY = hydrogen yield mol H_2 /mol glucose; Ref = name/s and date of author/s

5.3.1 The Effect of HRT on biohyhdrogen production

This study demonstrated that the sharp increase in hydrogen production rate (HPR) seem to be strongly influenced the HRT (Figure 4.8) when the HRT was reduced from 10h to 1.67h both the hydrogen production rate and yield increased (figure 4.9). Hydrogen content increased from 38% to 55% at HRT 2. 08 h (Figure 4.9). However, further reduction of HRT < 1.67 h demonstrated a very low substrate conversion efficiency of 55%. In addition, due to gas formation around granules, gas formation forced the granules to float (gas hold up) and accumulated towards the liquid surface on the top of the bioreactor (resulting in microbial biomass washout, at very low HRT < 1.67 h). Operating the bioreactor at short HRTs could be used to select for H_2-producing bacteria as compared to methanogenic bacteria. This is because the specific growth rate of methanogens is much shorter than those of H_2-producing bacteria (0.0167 and 0.083 h^{-1}, respectively). In general, if H_2-producing bacteria population can be stably maintained in the bioreactor against increase in hydraulic dilution arising from a decrease in HRT, the HPR and hydrogen content should increase with decrease HRT. This explains why HPR increased when the AFBR was operated at a progressively decreasing HRT from 10 h to 1.67 h.

5.3.2 The Effect of effluent recycle rate

In this study thermodynamic constrains preventing achievement of high HPs and HYs in a bioreactor with a high microbial biomass density were overcome by: Efficient removal of H_2 from the bioreactor by physical means, this was achieved by operating the bioreactor at high rates of de-gassed effluent recycling and improved design of the gas disengager. From the thermodynamic point of view, our experimental findings confirm that the application of external work in the form of high temperatures, high dilution rate and de-gassed effluent recycling can remove the thermodynamics constrains preventing the achievement of high HPs and HYs (Figure 5.1). It must be emphasized that thermophilic temperatures raises the chance of achieving the goal of economical H_2 production.

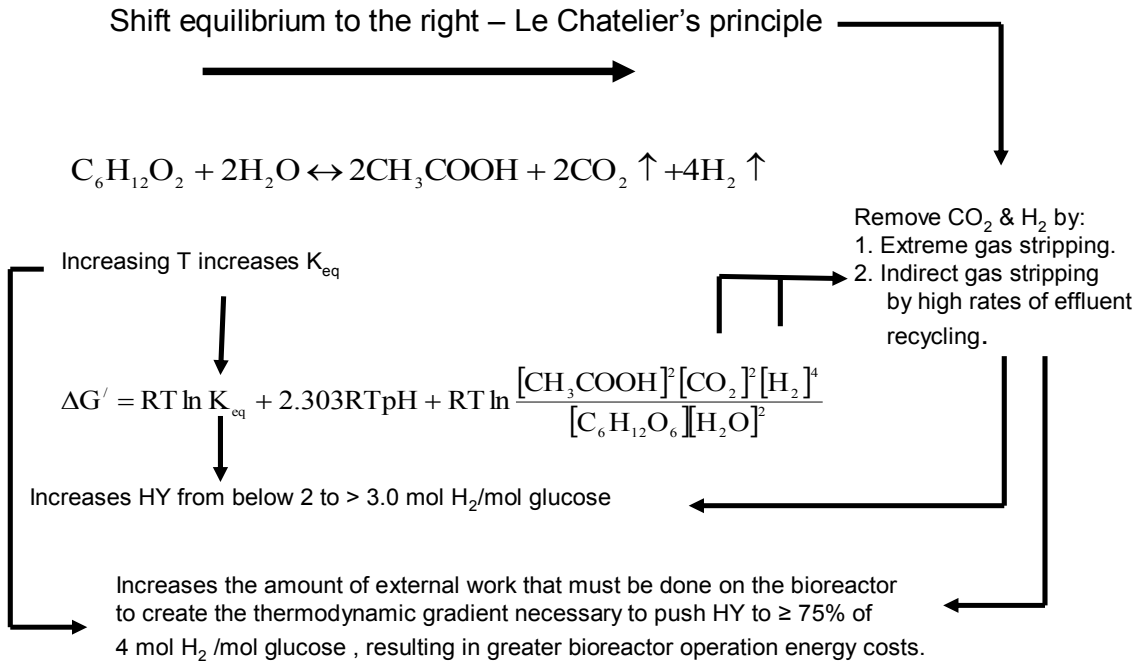

Figure 5.1. Gibb's free energy balance corresponding to bioreactor's HY.

5.3.2.1 Hydrogen partial pressure

Methods of reducing hydrogen partial pressure were studied before (Schnitzhofer et al., 2007). The high concentration of hydrogen within the bioreactor and dissolved liquid can results to metabolic shift to more fermentation end products (Angenent., 2004). Therefore, the influence of hydrogen partial pressure within the anaerobic H_2 production process is inevitable and is considered as an important approach towards improvement of hydrogen productivity (Mandal et al., 2002). In this study, rapid removal of H_2 produced within the bioreactor bed and the gas-desengager was promoted by gas stripping. Efficient removal of H_2 from the bioreactor was achieved by means of recycling of de-gassed effluent at a high flow rate through the bioreactor bed.

van Niel., (2003) reported that hydrogen production by thermophilic bacterium was inhibited at hydrogen pressures above 20 kPa, and a metabolic shift to lactate production was observed. Interestingly, it seems that there is a strong correlation between thermophilic temperatures, low hydrogen partial and the concentration of volatile fatty

acids in the reactor. For hydrogen yield (HY) above the theoretical threshold of 4.0 mol H_2/ mol glucose would require the anaerobic oxidation of acetate, butyrate and propionate in the absence of H_2 consuming bacteria. Under suitable thermodynamic conditions characterized by thermophilic temperatures > 50^oC and H_2 partial pressures < 20 Pa, the syntrophic anaerobic oxidation of acetate, propionate and butyrate is facilitated, see appendix 1.1, 1.2 and 1.3, respectively. In this study, the reduction of hydrogen partial pressure was achieved by hydrogen mass transfer from liquid to gas phase which was facilitated by combination of high effluent recycling rate and well design bioreactor gas-disengager.

5.4 A Relationship between hydrogen and soluble metabolites

Glucose fermentation can produce different end products, associated with different amount of hydrogen. Both acetic acid and butyric acid are well known as metabolites in the anaerobic H_2 fermentation of carbohydrates, and 4 mol and 2 mol of hydrogen were theoretically produced via the acetic acid and the butyric acid pathways, respectively. In contrast, propionic acid is considered as an undesirable product of H_2 fermentation (Chang et al, 2002).It is very important to quantify fermentation end products known as volatile fatty acids (VFAs), this is done in order to monitor their role in metabolic shifting from acidogenesis (H_2 production) to solventogenesis (production of acetone, ethanol, propanol or butanol) in *clostridia* which reduces hydrogen yield (Levin et al., 2004). Hence, VFA concentration distribution and their fractions are useful indicators for monitoring H_2 production.

In this study, three main VFAs were investigated (Acetate, propionate and butyrate) and ethanol as solvent was also monitored. As shown in table 4.2, acetic acid was the main major VFA component, and this confirmed that H_2 production belong to the acetate-type fermentation. In dark fermentation the metabolic pathway favoring acetate production is essential for efficient H_2 production. The principle being that NADH is usually generated by catabolism of glucose to pyruvate through glycolysis. The conversion of pyruvate to ethanol, butyric acid involves oxidation of NADH. The concentration of NADH would be

increased if the formation of these metabolites could be blocked or reduced. This will no doubt enhance H_2 production through the oxidation of NADH.

It is normally expected in glucose fermentation that more acetate or butyrate generated more H_2 produced. However, this is not the case with this study. In this study, the results showed that the concentration of acetate, propionate and butyrate declined from 16.01 mM, 6.43 mM and 6.42 mM to 5.19 mM, 2.05 mM and 1.31 mM, respectively (As shown in table 4.2). Interestingly, as concentration of VFAs decreased both the hydrogen content and hydrogen yield increased. In comparison, Koskinen et al., (2008) reported that a decrease in HY yield in their study was associated with increased lactate and ethanol, and a decrease in acetate and butyrate. This suggests that there is a correlation between the hydrogen yield and volatile fatty acids and solvent production. In this study several hypothesis were suggested to explain the decline in amount of VFAs while the HY increases. First hypothesis, in mixed microbial community during a fermentative process for H_2 production, under favorable thermodynamic bioreactor conditions microbial metabolic shift that favors high hydrogen production and yield will take place, thus producing more hydrogen. Second hypothesis is that there are bacteria through unusual pathway produce hydrogen, such as *acetogens*. Even though acetate oxidation is well known pathway, production of hydrogen as end product via this pathway is hardly reported in dark fermentation. It is also possible that the combined effect of low HRTs, high dilution recycle rates of de-gassed and operating the bioreactor at thermophilic temperatures created conditions that were favorable for the syntrophic oxidation acetate, butyrate and propionate (Schröder et al., 1994). The above mentioned phenomenon requires further investigation and the suggested pathways for the thermophilic anaerobic oxidation of acetate, butyrate or propionate (see appendix 2.1, 2.2 and 2.3, respectively).

5.4.1 A relationship between pH and soluble metabolites

Another important factor in metabolic shifting is pH, the monitoring and control of the pH in a H_2 producing reactor is important not only for the control of metabolic pathways (Lay, 2000) but also because pH serves as an inhibition mechanism for *methanogens* (Hwang et al., 2004). The choice of pH is important not only for the optimal production

of H_2, but also for the production of volatile fatty acids (VFA) and control of bacterial biomass growth. The accumulation of VFAs causes rapid drop in pH which is unfavourable to H_2 production. Moreover, some VFA can be toxic or inhibitory to the H_2-producing microbial population (Zheng and Yu, 2005). As discussed by van Ginkel and Logan, (2005) butyric acid could be more toxic than acetic acid in H_2 fermentation process, although there is no agreed threshold value for shifting from acidogenesis to solventogenesis. The optimum pH reported for solventogenesis is around 4.5 while for acidogenesis, it is 5.5 or higher (Ginkel et al., 2001; Ferchichi et al., 2005; Jones and Woods, 1986). In this study, hydrogen production rate and yield increased with simultaneous increase in pH from 4.5 to 7.09, after day 3 (Figure 4.13). These observations were consistent with the decrease in all values of the concentration of VFAs. These results suggest that a change in pH value leads to the change in fatty acids concentration or composition thus driving more NADH for the formation of hydrogen. Importantly, a change of pH in fermentation system causes the shift of bacterial metabolites, and the carbon flux at high pH value has more trends to production of more acetate and eventually, results increased hydrogen production.

5.4.2 Syntrophic microcolony model and VFAs

According to syntrophic microcolony model, a close synergistic relationship among different microbial groups is essential for efficient breakdown of the complex organic compounds. In fact, syntrophic microcolonies provide kinetics and thermodynamic requirements for intermediate transference and therefore efficient conversion (Schink and Thauer, 1988). Synergistic requirements would drive bacteria to form granules, in which different species function in a synergistic way and can easily survive. This model in H_2 production process is associated with improved or more efficient substrate utilization. In addition the presence of individual species may provide with crucial metabolic functional characteristics. These microorganisms can be easily obtained in natural environment where they co-exist, for example in sewage sludge.

In this study the model of syntrophic association among thermophilic microorganisms is shown in figure 5.2. The figure illustrates that sucrose substrate is converted by hydrogen producing microorganisms within the granule boundary layer and organic acids (acetate) are produced in the process, the presence of *acetogens* within the granule boundary layer could facilate oxidation of acetate top H_2 under favorable bioreactor conditions (e.g very low hydrogen partial pressure). VFAs which are produced are transported by random diffusion in all directions and thus penetrate the *acetogens* cluster within the granule, and thus converted to hydrogen. Since no methane was detected in this study, meaning *methanogens* were successfully inhibited. The produced H_2 is stripped away from the granule by high de-gassed effluent recycle rate from the bulk liquid. Indeed, in this study the effect of increased effluent recycle rate, thermophilic temperature and increased bacterial biomass resulted to thermodynamically favorable conditions within the reactor that facilitated high hydrogen production rate and yield.

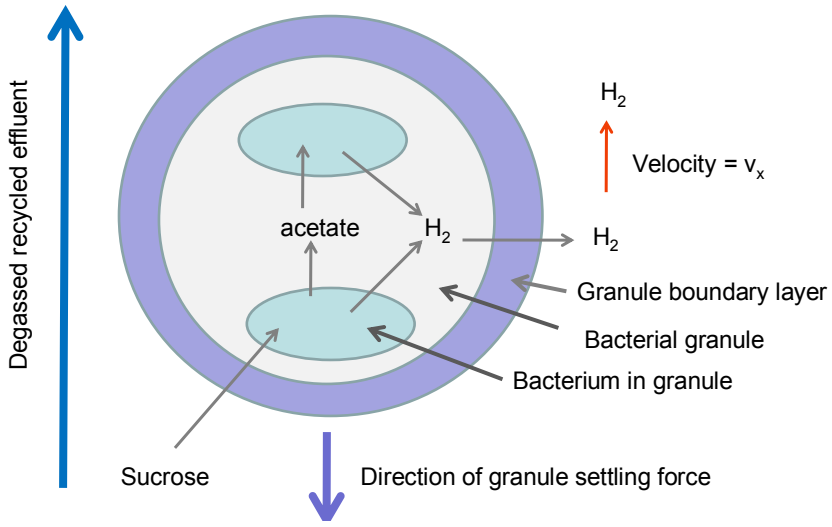

Figure 5.2: Anaerobic oxidation of sucrose and volatile fatty acids by syntrophic bacterial consortium in a granule

5.5 Morphological observation of microbial community

Scanning electron microscope (SEM) images shown in figure 4.6 and 4.7, reveal that there exist a porous structures on the surface of the hydrogen producing granule. The porous structure is likely to facilitate the passage for nutrients and substrate as well as release of hydrogen within structures of the granule. The granules were predominantly composed of rod-shaped hydrogen producing bacteria. As shown in figure 4.7b, the granules were strongly attached to each other by polymeric network called extracellular polymeric substances (EPS). EPS originates from biological synthesis and are localized on the outer surface of the bacteria and this structure plays a crucial role in the formation of granules. The yield of EPS has been reported to be strongly affected by carbon source and/or the variety of the granule microflora (Morgan et al., 1990). Furthermore, EPS serves as a bio-glue to facilitate agglutination of bacteria, the loosely adhered bacterial aggregates are strengthened by extra-cellular polymers secreted by bacteria to form firmly attached granule. The role of EPS in microbial granulation has been previously reported by (Hulshoff Pol et al., 2004; Liu et al., 2004). Furthermore, gram staining results were consistent with the results obtained from SEM images, rod shaped microorganisms were the most dominant organisms in the bioreactor, and also figure 4.5 reveals that the bacterial culture was a spore-forming microorganism.

5.6 Microbial species analysis

The denaturing gradient gel electrophoresis with 16S rDNA gene-targeted polymerase chain reaction (PCR) analysis was employed to study the bacterial composition present in the bioreactor. The results of 16S rDNA-targeted PCR-DGGE analysis confirmed the existence of dominant clostridia, as the main hydrogen producers in our hydrogen-producing system. The dominant bacterial strains in the reactor at HRT 2.08 h were C*lostridium thermopalmarium* and *Clostridium thermobutyricum* and *Clostridium botulinum* strain, the results also supported heating treatment of cell culture before inoculation as an effective method for enriching endospore-forming clostridia species. Other microorganisms detected were *Clostridium butyricum* and *Clostridium pasteurianum* and *Klepsiella sp,* which were observed at initial stages of bioreactor

operation but their composition changed as the reactor HRT was decreased from HRT 10 to 2.08 h. The presence of members of genus *Clostridium sp.* is consistent with reports in literature, where *clostridium* sp. is known for evolving hydrogen during anaerobic fermentation from different substrates (Karube et al., 1982; Wang et al., 2008; Fang et al., 2002). The presence of two clostridia species such as (*Clostridium butyricum and Clostridium pasteurianum*) are usually mesophilic. However, it was found that these isolates could grow at 65ºC. In addition, the *Klepsiella species* are also grew at 65ºC.

It is difficult to distinguish target bacterial strains in the mixed hydrogen producing system using PCR-DGGE technique. From PCR-DGGE profile established in this study, it was evident that some of the bacterial strains could not be detected, especially those that have low intensity bands (B, E, G, H and J). In comparison, other several studies which reported similar findings include (Liu et al., 2002; Hung et al., 2007). This might be due to the fact that other bacterial populations present in the bioreactor are present in small quantities and could not be detected by PCR-DGGE analysis, especially *acetogens.* So, microbial analysis by DNA-based techniques alone was insufficient for explaining the deviation of hydrogen-producing rates at different HRTs.

There is a need to develop a reliable technique for the determination of microbial population in the mixed consortia. In this study the use of undefined mixed cultures for fermentative hydrogen production is encouraged, however for a true reflection of microbial population in the reactor additional step is required, for example cloning technique. Cloning technique has proven to be a sophisticated tool for molecular DNA studies, and it is strongly suggested that it should be used as additional step in future studies.

5.7 SUMMARY OF ACHIEVEMENTS IN THIS STUDY

The AFBR system that we have developed facilitates the high rates of biohydrogen production at the maximum possible thermodynamic efficiencies. The design and operation of the AFBR system facilitates the following essential features for biohydrogen generation.

- High granular bacterial biomass retention under extremely high dilution rates or high linear fluid flow velocities (> 0.07 m s^{-1}).
- High bacterial biomass densities (35.1 g DM/L) within the bioreactor facilitate high volumetric reaction capacity.
- Also high organic loading rates (OLRs) in conjunction with the retention of high bacterial biomass densities result in the bioreactor having a high work rate with respect to the production of chemical energy in the form of hydrogen (32.7 L H_2/h).
- Hydrogen yields > 3.9 mol H_2/ mol glucose can be achieved as a result of the rapid rates of dissolved H_2 dilution within the fluidized granular bed due to either extremely high influent flow velocity or extremely high degassed effluent flow recycle velocities.

5.8 CONCLUSIONS

The experimental results indicated that through the appropriate bioreactor design and improved operational strategy, in conjunction with the suitable bacterial consortium, conditions could be achieved whereby H_2 partial pressure within the bioreactor could be reduced to levels necessary for achieving HYs equal to 4.0 mol H_2/mol glucose and HPRs equal to 32.7 L H_2/h. It was also demonstrated that thermophilic temperatures and high rates of degassed effluent recycling provided the external work that was necessary for overcoming the thermodynamic constraints preventing the simultaneous attainment of both HPRs and HYs. In addition, development of a modified fluidized bed bioreactor based on bacterial granules allowed the biohydrogen-producing system to be operated at a short HRT of up to 1 h.

5.9 FUTURE RESEARCH AND SUGGESTIONS

- Bacterial granulation was successfully induced from a variety of inoculum sources in this study. However, in some cases while working with one bioreactor it was not always easily to establish what factor or combinations of factors were critical for initiation of bacterial granulation. So, there is a need to understand all critical factors that control the successful reproducible induction of granulation.

- It will be interesting to understand more completely the microbial composition within the thermophilic biohydrogen producing bioreactor, especially on the basis of microbial syntrophic H_2 transfer interaction, including the role of microbial communities involved in the anaerobic oxidation of short chain fatty acids (includes acetate, propionate and butyrate oxidizing syntrophic bacteria). In light of this it is important to establish a reliable protocol to monitor the microbial species composition shift in the bioreactor, it was evident in this study that 16S rDNA PCR-DGGE technique was not sufficient to establish the complete species composition of bacterial community making up the granules. Therefore, an additional molecular technique such as molecular cloning is recommended in conjunction with PCR-DGGE technique to study microbial composition in the reactor. This will turn lead to an enhanced understanding of microbial activities in the reactor.

- A key feature of the bioreactor represented in figure 3.1 is the effluent gas disengager. Efficient removal of H_2 from the liquid phase of the bioreactor could be achieved by means of recycling of de-gassed effluent at a high flow rate through bioreactor bed. However, in this study the concentration of hydrogen partial pressure within the recycled liquid bulk phase was not determined. It is therefore, recommended that in future studies the exact and accurate level of hydrogen dissolved in recycle liquid bulk must be investigated, in order to completely satisfy that the application of high effluent recycle rates can remove the thermodynamic constrains preventing the simultaneous achievement of high hydrogen yield and productivity.

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APPENDICES

Appendix 1- Oxidation of volatile fatty acids and hydrogen partial pressure

Appendix 1.1: The effect of H_2 partial pressure and temperature on ΔG' for anaerobic acetate oxidation.

Hydrogen Partial Pressure (Pa)

T	ΔG°'	0.1	1	5	10	20	30	60
25	104.76	-37.21	-14.38	1.58	8.45	15.33	19.35	26.22
30	102.49	-41.86	-18.64	-2.42	4.57	11.56	17.08	24.90
35	100.13	-46.60	-23.00	-6.51	0.59	7.70	11.85	18.95
40	97.77	-51.34	-27.36	-10.60	-3.38	3.83	8.06	15.28
45	95.41	-56.08	-31.72	-14.69	-7.36	-0.03	4.26	11.60
50	93.05	-60.83	-36.08	-18.79	-11.34	-3.89	0.47	7.92
55	90.68	-65.57	-40.44	-22.88	-15.31	-7.75	-3.32	4.24
60	88.32	-70.31	-44.80	-26.97	-19.29	-11.61	-7.12	0.56
65	85.96	-75.05	-49.16	-31.06	-23.27	-15.47	-10.91	-3.12
70	83.60	-79.80	-53.52	-35.15	-27.24	-19.33	-14.71	-6.80

Gibbs Free Energy (kJ/mol)

Acetate = 50 mM
HCO_3^- = 84 mM
H^+ = pH 7.0

Minimum quantum of Gibbs free energy for ATP production «-20 kJ/mol

Bioreactor operational parameters for thermodynamic feasibility for dark anaerobic acetate oxidation

Appendix 1.2: The effect of H2 partial pressure and temperature on ΔG' for anaerobic propionate oxidation.

Hydrogen Partial Pressure (Pa)

T	ΔG°'	0.1	1	5	10	20	30	60
25	76.30	-38.94	-21.81	-9.85	-4.69	0.46	3.48	8.63
30	74.39	-42.78	-25.37	-13.20	-7.96	-2.72	1.57	5.59
35	72.44	-46.66	-28.97	-16.60	-11.27	-5.94	-2.83	2.50
40	70.48	-50.55	-32.57	-20.00	-14.58	-9.17	-6.00	-0.59
45	68.53	-54.44	-36.17	-23.40	-17.90	-12.40	-9.18	-3.68
50	66.57	-58.33	-39.77	-26.80	-21.21	-15.62	-12.35	-6.77
55	64.62	-62.21	-43.37	-30.20	-24.52	-18.85	-15.53	-9.86
60	62.66	-66.10	-46.97	-33.59	-27.84	-22.08	-18.71	-12.95
65	60.71	-69.99	-50.57	-36.99	-31.15	-25.30	-21.88	-16.04
70	58.75	-73.88	-54.17	-40.39	-34.46	-28.53	-25.06	-19.13

Gibbs Free Energy (kJ/mol)

Propionate = 50 mM
Acetate = 4 mM
HCO_3^- = 84 mM
H^+ = pH 7.0

Minimum quantum of Gibbs free energy for ATP production « -20 kJ/mol

Bioreactor operational parameters for thermodynamic feasibility for dark anaerobic propionate oxidation

Appendix 1.3: Affect of H2 partial pressure and temperature on ΔG' for anaerobic butyrate oxidation.

		Hydrogen Partial Pressure (Pa)						
T	ΔG°'	0.1	1	5	10	20	30	60
25	48.24	-42.60	-31.18	-23.20	-19.76	-16.33	-14.32	-10.88
30	44.93	-47.43	-35.82	-27.71	-24.22	-20.72	-17.63	-10.88
35	41.62	-52.26	-40.47	-32.22	-28.67	-25.12	-23.04	-19.49
40	38.31	-57.10	-45.11	-36.73	-33.12	-29.51	-27.40	-23.79
45	35.00	-61.93	-49.75	-41.24	-37.57	-33.90	-31.76	-28.09
50	31.68	-66.77	-54.39	-45.75	-42.02	-38.30	-36.12	-32.39
55	28.37	-71.60	-59.04	-50.26	-46.47	-42.69	-40.48	-36.70
60	25.06	-76.44	-63.68	-54.76	-50.93	-47.09	-44.84	-41.00
65	21.75	-81.27	-68.32	-59.27	-55.38	-51.48	-49.20	-45.30
70	18.44	-86.10	-72.97	-63.78	-59.83	-55.87	-53.56	-49.60

Gibbs Free Energy (kJ/mol)

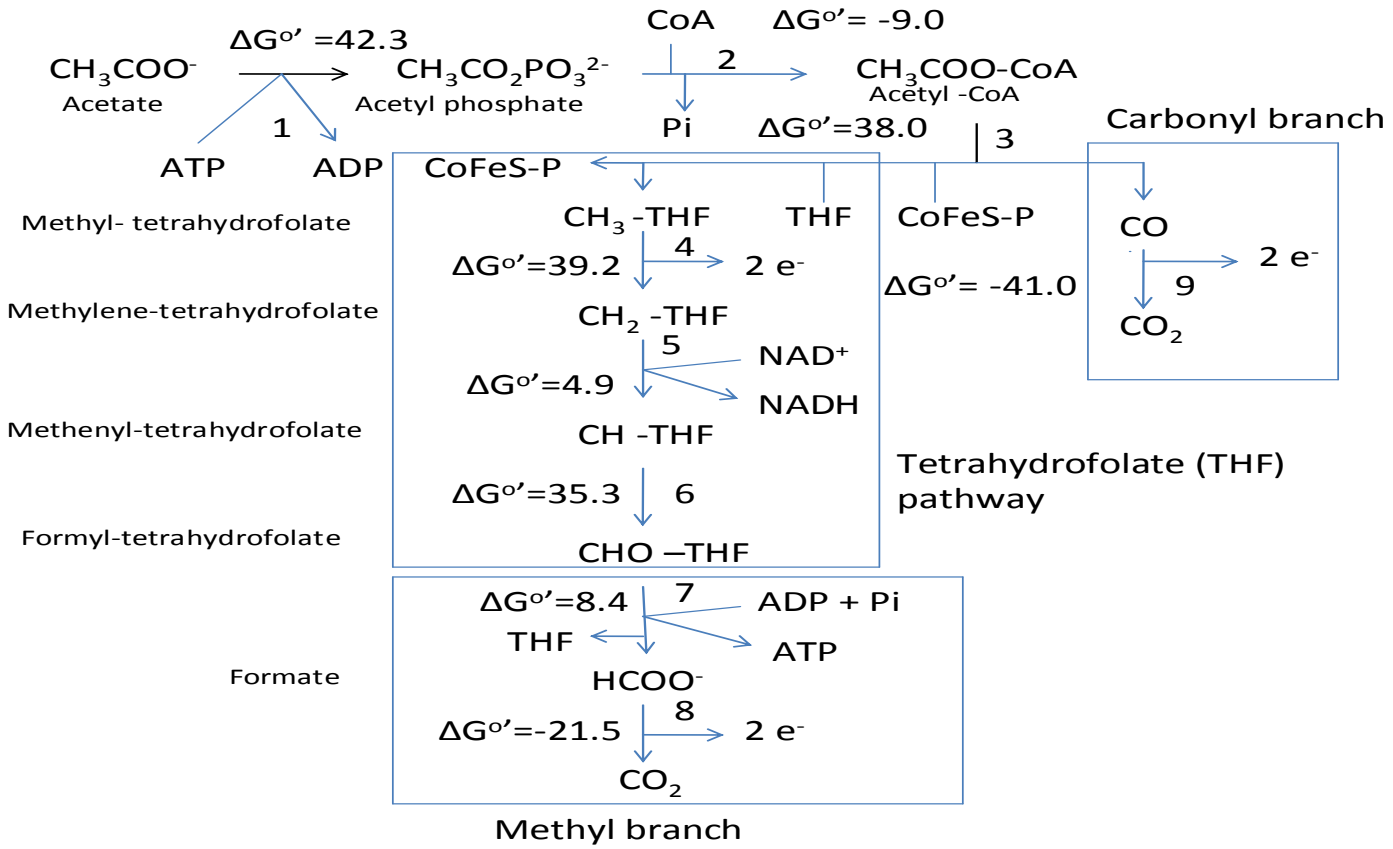
Butyrate = 50 mM
Acetate = 4 mM
HCO_3^- = 84 mM
H^+ = pH 7.0

Minimum quantum of Gibbs free energy for ATP production « -20 kJ/mol

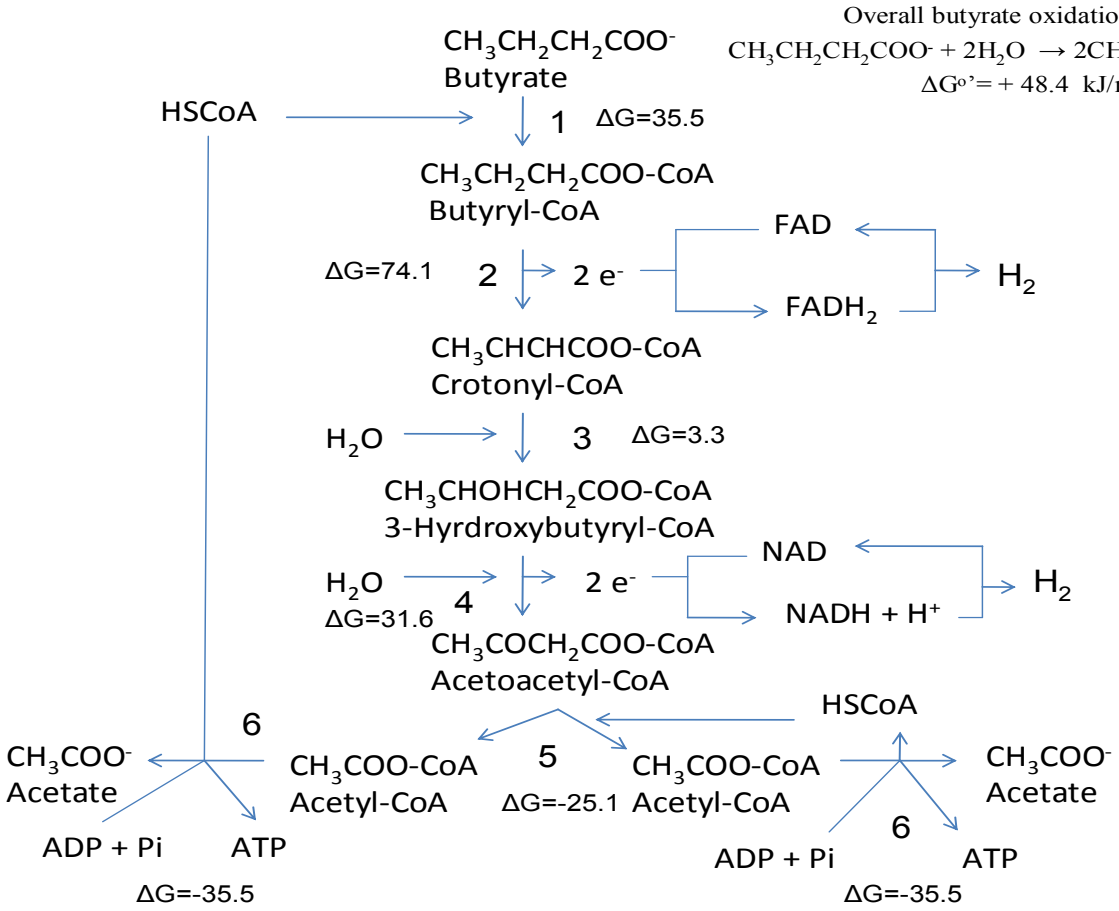
Bioreactor operational parameters for thermodynamic feasibility for dark anaerobic acetate oxidation

Appendix 2- Oxidation of volatile fatty acids metabolic pathways

Appendix 2.1: Acetate oxidation or acetate degradation pathway



Appendix 2.2: Butyrate degradation or butyrate oxidation pathway



Enzymes involved:

1 – butyryl-CoA:acetate CoA transferase

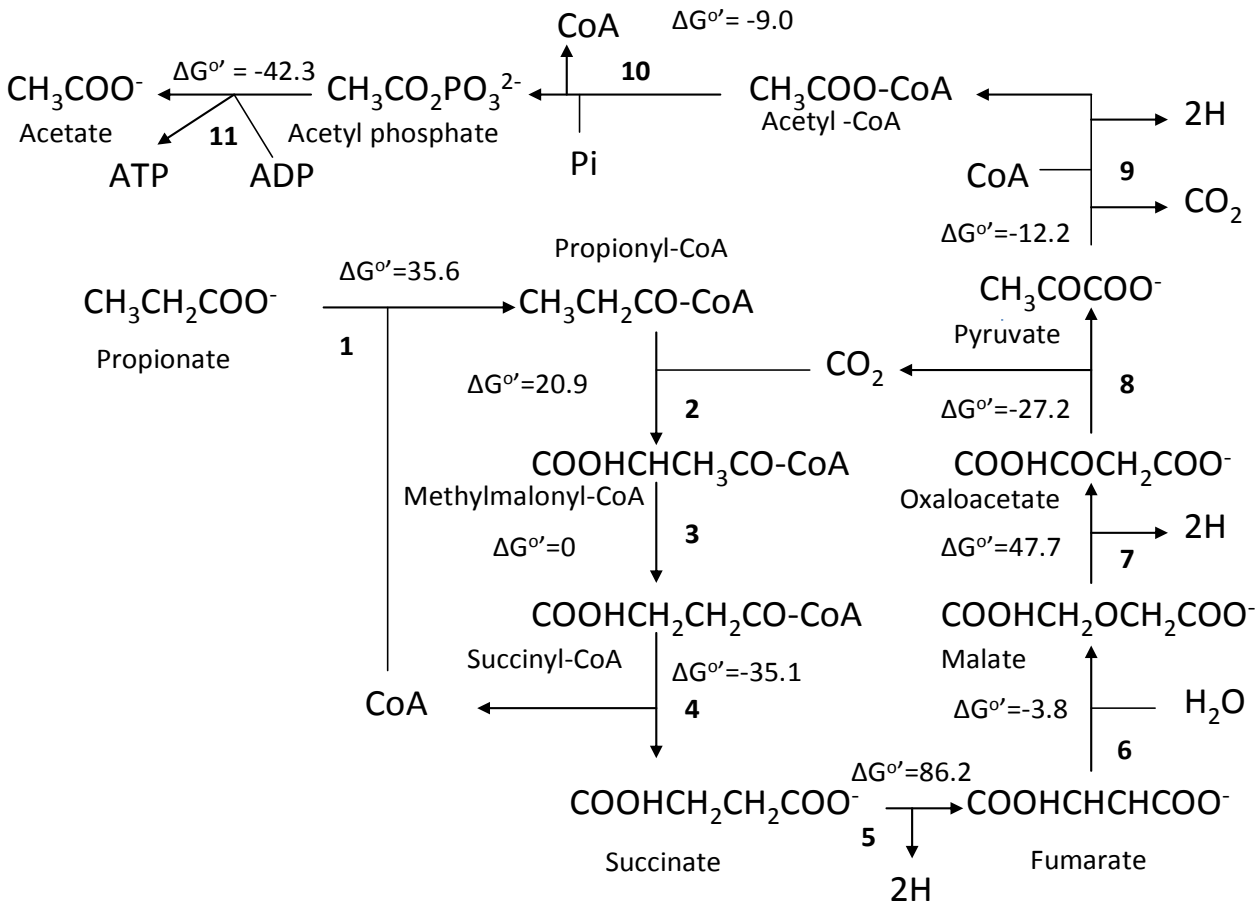
2 – acyl-CoA dehydrogenase

3 – enoyl-CoA hydratase

4 – hydroxylbutyryl CoA dedydrogenase

5 – acetyl-CoA acetyltransferase

Appendix 2.3: Overall propionate or propionate degradation pathway



Appendix 3: 16S rDNA sequences for all bacterial bands isolated in the bioreactor

Bioreactor isolate: B06_co 1, Band I

TTCGGGGCAGGAAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAG
ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCGTTAGTTGCTACCAT
TAAGTTGAGCACTCTAACGAGACTGCCGCGGTTAACGTGGAGGAAGGTGGGG
ATGACGTCAAATCATCATGCCCCTTATGTCTAGGGCTACACACGTGCTACAAT
GGCCGGTACAACGAGATGCAAACCCGTGAGGGGGAGCCAAACTTCAAAGCC
GGTCCCAGTTCGGATTGTAGGCTGAAACTCGCCTACATGAAGTCGGAGTTGC
TAGTAATCGCGAATCAGCATGTCGCGGTGAATACGTTCCCGGGTCTTGTACAC
ACCGCCCGTA

Bioreactor isolate: A03_PCM3, Band C

CGTAGAGATACGTGAAGCCCTTCGGGGCAGGAAGACAGGtGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTATCATTAGTTGCTACCATTAAGTTGAGCACTCTAGTGAGACTGCCCGGGT
TAACCGGGAGGAAGGCGGGGATGACGTCAAATCATCATGCCCCTTATGTCTA
GGGCTACACACGTGCTACAATGGTGAGAACAACGAGATGCAATACCGCGAG
GTGGAGCCAAACTTGAAAACTCATCCCAGTTCGGATTGTAGGCTGAAATTCG
CCTACATGAAGTTGGAGTTGCTAGTAATCGCGAATCAGAATGTCGCGGTGAA
TACGTTCCCGGGCCTTGCACACACCGCCCGTAC

Bioreactor isolate: F01_PCM 1, Band A

GTGGTCGGTTCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCTTATGTTGCCAGCACATTA
TGGTGGGTACTCATGAGAGACTGCCGGGGTTAACTCGGAGGAAGGTGGGGAT
GACGTCAAATCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGG
TCGGTACAATGAGATGCAACCTCGCGAGAGTGAGCAAAACTATAAAACCGAT
CTCAGTTCGGATTGTAGGCTGAAACCCGCCTGCATGAAGTTGGAATTGCTAGT
AATCGCGGATCATAATGCCGCGGTGAATACGTTCCCGGGCCTTGTAA

Bioreactor isolate: G06_LP2, Band D

GGGTTCACCGATTCCAACTTCTGGAGTCAAGTTGCAAACTCCAATCCGAACTA
CAACGTATTTTATAAGGTCCGCTTGCTCCCCCAAGGTCGCTTTCTTTTGTTTCC
GCCATTGTAGCACGGGGGTAGCCCTGGTCGTAAGGGCCATGATGACTTGACG
YCATCCCCCCCTTCCCCCAGTTTATCCCTGGCAGYCCCCTTTGATTTCCCGGC
CGAACCGCTGGCAACAAAGAATAAGGGTTGCGCTCGTTGCGGGACTTAACCC
AACATTTCACAACCCAAGCTAACAACAACCATGCACCACCTGTCTCACAGTT
CCCAAAGGCCCCAATCCATCTCTGAAAATTTCTGTGAATGTCAAAACCAGGA
AAGGTTCTTCGCGTTAAA

Appendix 4 - Media preparations and buffers

The composition of media

A modified Endo medium formulation (Endo et al., 1982) used in this study with some modifications.

Chemical Components	g/L
Sucrose	17.63
$NaHCO_3$	3. 36
NH_4HCO_3	3.490
$MnSO_4$	0.015
$CaCl_2$	0.2
K_2HPO_4	0.699
$NaHCO_3$	3.36
$MgCl_2\ 6H_2O$	0.015
$FeSO_4\ 7H_2O$	0.0225
$CuSO_4\ 5H_2O$	0.005
$CoCl_2\ H_2O$	1.24×10^{-4}

Gel electrophoresis

Agarose Gel (50 ml)

Agarose (0.5g for 1%)

10ml 5X TBE

40ml distilled water

Heat until agarose has completely dissolved

Add 1 µl Ethidium Bromide

5X TBE

54g Tris base

27.5g Boric acid

20ml 0.5M EDTA pH 8.0

Make up to 1L with distilled water and autoclave at 121ºC at 15psi for 20 minutes

Electrophoresis buffer

100ml 5X TBE

900ml sterile distilled water

2.5 µl Ethidium Bromide

10% Ammonium persulfate (APS)

APS 100mg

dH_2O 1ml

Store at -20ºC

TE Buffer

10 mM Tris-HCl, pH 8.0

1 mM EDTA

Sterilize solutions by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle.

Store the buffer at room temperature.

Appendix 5: Provisional patent application receipt

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978
APPLICATION FOR A PATENT AND ACKNOWLEDGEMENT OF RECEIPT
[Section 30(1) – Regulation 22]

Form P1

REGISTRAR OF PATENTS, DESIGNS, TRADE MARKS AND COPYRIGHT
FILED
Revenue stamps or revenue franking machine impression
REGISTRATEUR VAN PATENTE, MODELLE, HANDELSMERKE EN OUTEURSREG

The granting of a patent is hereby requested by the undermentioned applicant in the terms of the application filed in duplicate

Official Application No.: 21 01 2010/04063

Applicant's or agent's reference: CAVENY

(ii) 71 Full name(s) of applicant(s): University of the Witwatersrand, Johannesburg

(iii) 1 Jan Smuts Avenue, Johannesburg

Address(es) of applicant(s):

(iv) 54 Title of invention: Effluent Gas-discharger

(v) The applicant claims priority as set out on the accompanying form P2.

(vi) This application is for a patent of addition to Patent Application No. 21 01

(vii) The application is a fresh application in terms of section 37 21 01 and is based on Application No.:

(viii) This application is accompanied by:

X 1. A single copy of a provisional or two copies of a complete specification of 9 pages.
2. Drawings of sheets.
3. Publication particulars and abstract (form P8 in duplicate).
4. A copy of Figure of the drawings (if any) for the abstract.
X 5. An assignment of the invention.
6. Certified priority documents (state number).
7. Translation of the priority documents.
8. An assignment of the priority rights
9. A copy of Form P2 and the specification of S.A. Patent application No 21 01
X 10. A declaration and power of attorney on Form P3.
11. Request for ante-dating on Form P4.
12. Request for classification on Form P9.

(ix) Address for service: Dr R.J.Caveney, Wits Enterprise, Private Bag 3, WITS 2050

Dated this 7th day of June 2010

Signature of applicant(s) or agent

Received
Office of the Registrar of Patents
Registrar of Patents

REGISTRAR OF PATENTS, DESIGNS, TRADE MARKS AND COPYRIGHT
18 OCT 2010
REGISTRATEUR VAN PATENTE, MODELLE, HANDELSMERKE EN OUTEURSREG

The duplicate will be returned to the applicant's address for service as proof of lodging but is not valid unless endorsed with official stamp.

Appendix 6: Glossary of terms

Anaerobic digestion: Decomposition of biological wastes by micro-organisms, usually under wet conditions, in the absence of air (oxygen), to produce biogas.

Biofuel: Fuel produce directly or indirectly from biomass. The term biofuel applies to any solid, liquid, or gaseous fuel produced organic (once living) matter. The word biofuel covers a wide range of products, some of which are commercially available today, and some of which are still in the research and development phase.

Biogas: A combustible gas derived from decomposing biological waste under anaerobic condition. Biogas normally consists of 50-60% methane, 25-50% carbon dioxide, and other possible elements such as nitrogen, hydrogen or oxygen.

Biomass: Organic matter available on a renewable basis. Biomass includes forest and mill residues, agricultural crops and wastes, wood and wood wastes, animal wastes, livestock operation residues, aquatic plants, fast-growing trees and plants, and municipal and industrial wastes.

Bioreactor: A bioreactor is a vessel in which a biochemical process occurs. This usually involves organisms or biochemically active substances derived from such organisms.
Cellulose: Polysaccharides (long chain of simple sugar molecule) with formula $(C_6 H_{10}O_5)n$

Charcoal: Solid residue derived from carbonization distillation, pyrolysis, and torrefaction of fuelwood.

Combustion: The transformation of biomass fuel into heat, chemicals, and gases through chemical combination of hydrogen and carbon in the fuel with oxygen.

Digester: An airtight vessel or enclosure in which bacteria decompose biomass in wet condition to produce biogas.

Effluent: Effluent liquid or gas discharge from a process or chemical reactor, usually containing residues from that process.

EJ: Exajoules (1 EJ = 10^{18} J).

Emissions: waste substances released into the air or water.

Energy crops: Crops grown specifically for their fuel value. These include food crops such as corn and sugar-cane, and non-food crops such as poplar trees and switchgrass.

Feedstock: A feedstock is any biomass resource destined for conversion to energy or biofuel. For example, corn is a feedstock for ethanol production, soybeans oil may be

feedstock for biodiesel and cellulosic biomass has the potential to be a significant feedstock source to bioethanol.

Fermentation: Conversion of carbon containing compounds by microorganisms for production of fuels and chemicals such as alcohols, acids or energy-rich gases. It is a biochemical reaction that breaks down complex organic molecules (such as carbohydrates) into simpler materials (such as ethanol, carbon dioxide, and water). Bacteria or yeast can ferment sugars to bioethanol.

Fossil fuel: solid, liquid, or gaseous fuels formed in the ground after millions of years by chemical and physical changes in plant and animal residues under high temperature and pressure. Oil, natural gas, and coal are fossil fuels.

Greenhouse effect: The effect of certain gases in the Earth's atmosphere in trapping heat from the sun

Hydrocarbons: Any chemical compound containing hydrogen, oxygen, and carbon

Hydrogen: Simple molecule conceivable, with a molecular formula of H_2. Gaseous fuel that can be produced from fossil fuels, biomass and electricity.

Methane: Methane is a combustible chemical compound with the molecular formula CH_4. It is the principal component of natural gas.

Organic matter: Matter that comes from once living-living organism

Particulate: A small, discrete mass of solid or liquid matter that remains individually dispersed in gas or liquid emissions. Particulate take the form of aerosol, dust, fume, mist, smoke, or spray. Each of these forms has different properties.

Sludge: Sludge is formed in the reaction basin during biological waste water treatment process and separated by sedimentation. Sludges can be converted into biogas via anaerobic digestion.