Chapter 1
Literature Review

1.1 Introduction

In the early nineteenth century, the estimated population of the planet was about 1 billion people, with only 3% of the population which living in cities. Most the planet's energy base comprised human and animal power, supplemented with the use of renewable energy (Planethopia, 2011). It was not until the early twentieth century that, the use of fossil fuels gained prominence and shifted the global importance of the energy matrix. By 1900, the human population exceeded 1.6 billion inhabitants and the rate for urbanisation increased by up to 15%. The rate and scale of urbanisation was at its highest with Western Europe as the centre of the industrial revolution. It was during this time that the first cities with a population of over a million inhabitants emerged.

Today, on the threshold of the new century and millennium, the world population stands at over 7 billion people, more than half living in cities: over a hundred times more than in 1800. The energy base for modern cities is dominated by fossil fuel, constituting 80% of all energy sources; while renewable sources constitute only 6% renewable energy (McLamb, 2011; Planethopia, 2011). However, this urban industrial world faces two enormous challenges: The first is the depletion of fossil fuels and an increase in associated emission. The second challenge faced by modern societies is how to cope with an exponential increase of human population. In response to the two problems mentioned above, continuous effort has been made in exploration of clean, renewable alternatives for sustainable development (Ni *et al*., 2006).

1.1.1. Fossil fuels

Fossil fuels are the carbon rich-remains of prehistoric plants and animals to intense heat and pressure inside the earth over millennia. This results in the formation of coal, oil and natural gas. The current rate of the consumption of fossil fuels is not sustainable as it far exceeds the rate of replenishment (US EIA, 2009).

1.1.2 Fossil fuels dilemma

Combustion of fossil fuels is considered to be the largest contributing factor to the release of greenhouse gases into the atmosphere. Carbon dioxide (CO_2), water vapours (H_2O), methane (CH_4), nitrous oxide (N_2O), sulfur hexafluoride (SF_6) and chlorofluorocarbons (CFCs) are also called greenhouse gases (Warrick *et al*., 1993). These gases are relatively transparent to incoming solar radiation, allowing sunlight to pass through the atmosphere to the surface of the earth. The energy is then absorbed and warms the earth's surface and the rest is reflected back to the atmosphere as infrared radiation however, some is absorbed by greenhouse gas molecules. Due to this absorption, the energy is trapping additional heat in the lower atmosphere is illustrated in Figure 1.1. The higher the concentration of green house gases in the atmosphere, the more heat energy is being reflected back to the Earth (CCIR, 2005).

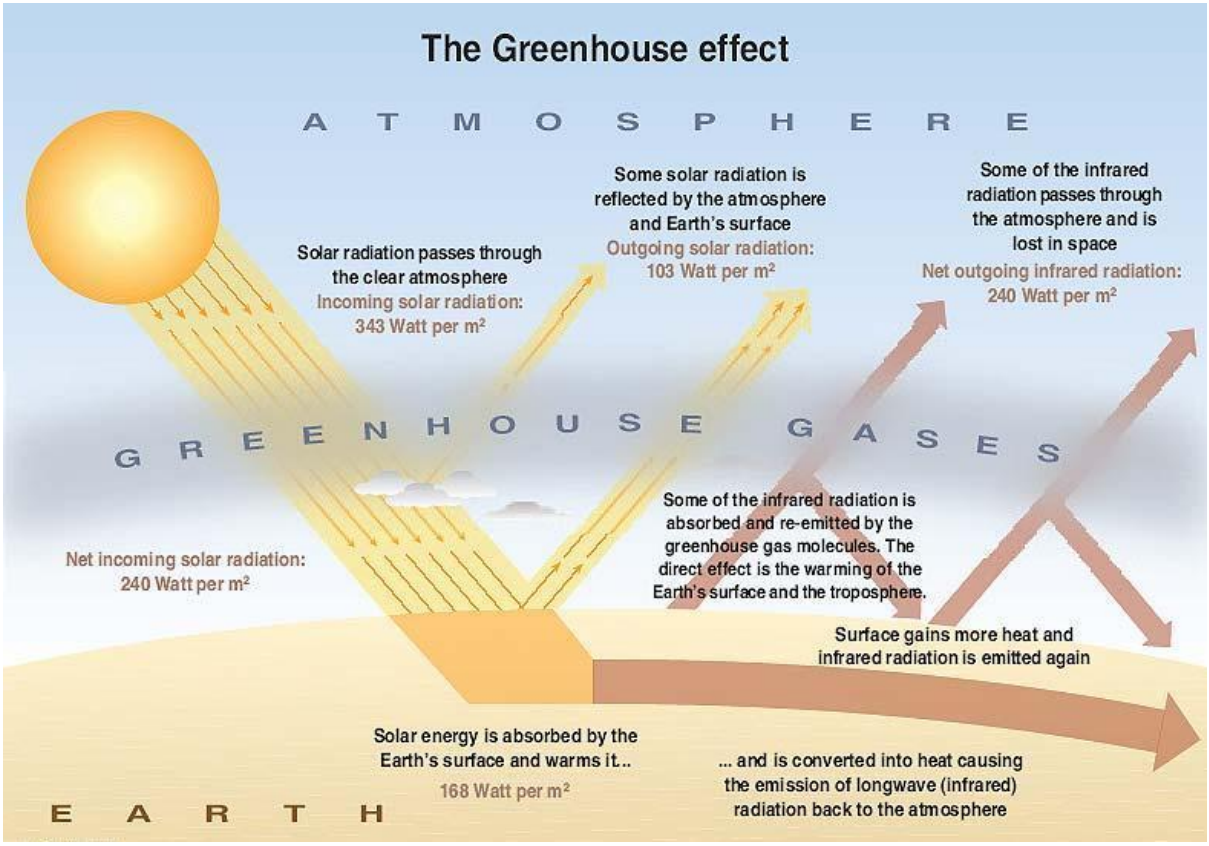

Figure 1.1 A schematic illustration of the greenhouse effect (Source Okanagan University college in Canada, Department of Geography; University of Oxford, School of Geography; United states Protection Agency (EPA) Washington, Intergovernmental Panel on Climate Change (IPCC) 1996).

1.1.3 Impact of global warming

A significant intensification of the greenhouse effect has serious implications for and on the global climate (WHO, 2010) through global warming. The impact of global warming on the environment is extensive and affects many areas: Human health, agriculture, natural ecosystems, coastal areas, and heating and cooling (McLamb, 2011).

1.1.3.1 Health

Climate experts are particularly confident that climate change will bring increasingly frequent and severe heat waves and extreme weather events. These changes have the potential to affect human health in several direct and indirect ways, some of them severe (CDP, 2010). Diseases such as malaria, dengue fever, yellow fever, encephalitis, algal blooms may occur more frequently as temperatures rise. The outcomes could be severe and result in increased illnesses and deaths. In addition, heat can increase air pollution, increasing the severity of respiratory diseases such as asthma and chronic obstructive pulmonary disease (CDP, 2010; US EPA, 2011).

1.1.3.2 Agriculture and food supply

Solar radiation, temperature, and precipitation are the main drivers of plant growth. Therefore, agriculture is highly sensitive to climate variability and weather extremes, such as droughts, floods and severe storms. Human activity has changed atmospheric characteristics by emitting large amounts of greenhouse gases into the atmosphere, resulting in higher global temperatures, affecting rainfall and increasing climatic variability (US EPA, 2011). Climate change is projected to have significant impacts on the production of staple foods in many of the poorest regions; and is expected to rise by up to 50% by 2020 in some African countries. Reduced agricultural productivity and yields as a result of climate change will increase the prevalence of chronic and acute child malnutrition, low birth weights and suboptimal breastfeeding, which currently cause deaths of 3.5 million mothers and young children every year (CI, 2008; WHO, 2010).

1.1.3.3 Ecosystems and biodiversity

Climate is an integral part of ecosystems, with organisms adapting to their regional climate over time. Substantial changes have already occurred in ecosystems resulting from human activities. Climate change on this scale, is recognised as an anthropogenic-induced phenomenon (Perrings, 2010; US EPA, 2011). The risk of extinction could increase for many species, especially those that are already endangered due to human development, low population numbers, narrow temperature tolerance range. This can therefore, alter ecological functions and life support services that are vital to the well-being of human societies (US EPA, 2011; UNEP, 2010).

1.1.3.4 Coastal Zones and Sea Level Rise

The warming of the earth will result in the melting of mass volumes of ice in the polar region and the thermal expansion of sea water which will ultimately result in a sea level rise. Such rise have significant impacts on the coastal ecology, inundate deltas, and other coastal lowlands, beach erosion, exacerbate coastal flooding, and threaten water quality in estuaries and aquifers (Theron and Rossouw, 2008; US EPA 2011). In Southern African region, recent observations from satellites, demonstrate that the sea level rise from 1993 to 2006 was 3.1+/- 0.7 mm/y (Cazenave and Neren, 2004) while the latest data give sea level rise of 3.3+/- 0.4 mm/y in 1993-2006 (Rahmstorf *et al*, 2007; Theron and Rossouw, 2008). The report released by intergovernmental panel on climate change (IPCC) 2007 predicts that seal level rise continues for centuries due to the timescales associated with climate processes and feedback. By 2100, the sea level will be in the range of 0.18-0.59 m. This will depend on the emission of gas.

1.1.3.5 Polar region

There is evidence that climate change is already having observable impacts in the Arctic and in the Antarctica. Many of these observed changes are consistent with the expected effects of climate change. In 2004, the Arctic Climate Impact Assessment (ACIA) report was released at the Symposium held in Reykjavik, Iceland. This report was produced by an international team of scientists at the request of the Arctic Council, the International Arctic Science Committee (IASC) and 8 nation intergovernmental forums including the United States. The ACIA concluded the following:

- The Arctic and Antarctic regions are warming faster than previously thought, raising world sea levels and making drastic global climate change more likely than ever;
- With rise in temperature, glaciers mountain are declining in both polar regions, affecting human livelihoods, local plant and animal life as well as global atmospheric circulation and sea-level.

1.1.3.6 Global renewable energy sources

The world energy consumption in 2005 stood at 488 EJ (exajoule, 1018 J) and has been estimated to exceed 650 EJ by 2025 with 86% supplied by fossil energy, but reliance on fossil fuels presents a big problem (Hasyim *et al*., 2010). They are limited in supply and will one day be depleted or will become too expensive to retrieve what is left. There is therefore a need for an alternative fuel energy source that will meet the energy demands of an increasing population in a sustainable and environmental manner (Kyazze *et al*., 2005). Renewable energy resources, such as solar energy**,** wind energy**,** geothermal power**,** hydropower**,** biomass, offer clean alternatives to fossil fuels.

1.1.3.7 Solar energy

Solar energy is an excellent alternative energy source that can be used for generating electricity, heating, cooling and lighting and a variety of industrial processes. This energy has many positive impacts on the environment: Firstly, it can operate independently, secondly, it is cost-effective, thirdly, it reduces the dependence on centralised sources of energy, influenced by natural disasters and fourthly, it creates absolutely no pollution in the atmosphere. Therefore, it does not contribute to global warming. These are perhaps the most important advantages that make solar energy much more practical than greenhouse gases in this technology (REN21, 2011). There are three ways of capturing, converting and distributing solar energy. The most commonly used system is solar thermal electrical which converts solar radiation in order to boil water for a turbine generator. In order to boil water using sunlight, one has to concentrate the light from a very large area into a very small area. This can be done with a magnifying glass. However, for a large system that is going, to create electricity, magnifying glasses are not useful, as such a large lens would have glass so thick that a great deal of the light would be absorbed by it. The second way is the Photovoltaic system which converts solar radiation directly into electricity. The disadvantage for these systems is the cost of making them. The solar cells and solar panel that are needed

to harness solar energy tend to be very expensive and dependent on the weather. Solar power cannot be used during a storm, on cloudy day or at night (ESA21, 2003).

1.1.3.8 Wind energy

Denmark was the World's biggest manufacturer of wind turbines until wind power became a popular source of CO_2 free electricity in the rest of the World. Wind turbine manufacture has since become widely dissipated outside Denmark (CEPOS, 2009). Today, many areas around the globe have started the switch to wind energy. This energy can be used in different ways: wind turbines and wind mills. A wind turbine is a rotating device which converts the kinetic energy from the wind into mechanical energy and this energy is used to generate electricity **(**McLamb, 2011). But if the energy is used directly, such as to pump water or to grind grain, the machine is most commonly referred to as a windmill. The main disadvantage of wind-generated power is the high level of variability and unpredictability of winds. In many areas, the wind's strength is too low to support a wind turbine or wind farm, and this is where the use of solar power or geothermal power could be potential alternatives (IEA, 2005).

1.1.3.9 Hydropower

In the late 19th century, hydropower became a source for generating electricity using the energy from moving water (INL, 2011). Water constantly moves through a vast global cycle, evaporating from lakes and oceans, forming precipitation as rain or snow, and then flowing back down to the oceans through rivers. The fall and movement of water is part of a continuous natural cycle. The energy of this water cycle can be tapped to produce electricity **(**McLamb, 2011). The capacity of such hydropower plant depends on the height that the water-falls and the quantity of water flowing. The greater the height of the water above the turbine, the more pressure the water can impart to spin the turbine, which drives the generator to produce the electrical power (IEA, 2005; WBG, 2009).

1.1.3.10 Geothermal power

In the 20^{th} century, the demand for electricity has led to the consideration of geothermal power as a generating source. The Earth's internal heat produces steam and hot water that can be used to power generators and produce electricity (IEA, 2007b). Geothermal energy has many advantages: It is clean, can be extracted without burning fossil fuel such as coal,

gas, or oil, is cost effective, uses less land per megawatt than for almost every other type of power plant, and does not damage the environment through dams or mining. Geothermal power have until recently, been built exclusively where high temperature geothermal resources are available, near tectonic plate boundaries (IEA, 2005; McLamb, 2011).

1.1.3.11 Energy from biomass

The need for an alternative fuel energy that will meet the energy demands of an increasing population, and also without no impacting on the environment is one of the main challenges that mankind will face over the coming decades, particularly because of the need to address climate change (Kyazze *et al*., 2005). Most leading international and national bodies have identified biomass as a good contender in this respect. It is presently the largest global contributor of renewable energy (Figure 1.2) and has significant potential to expand in the production of heat electricity and fuels for transport (Kyazze *et al*., 2005; Ni *et al*., 2006).

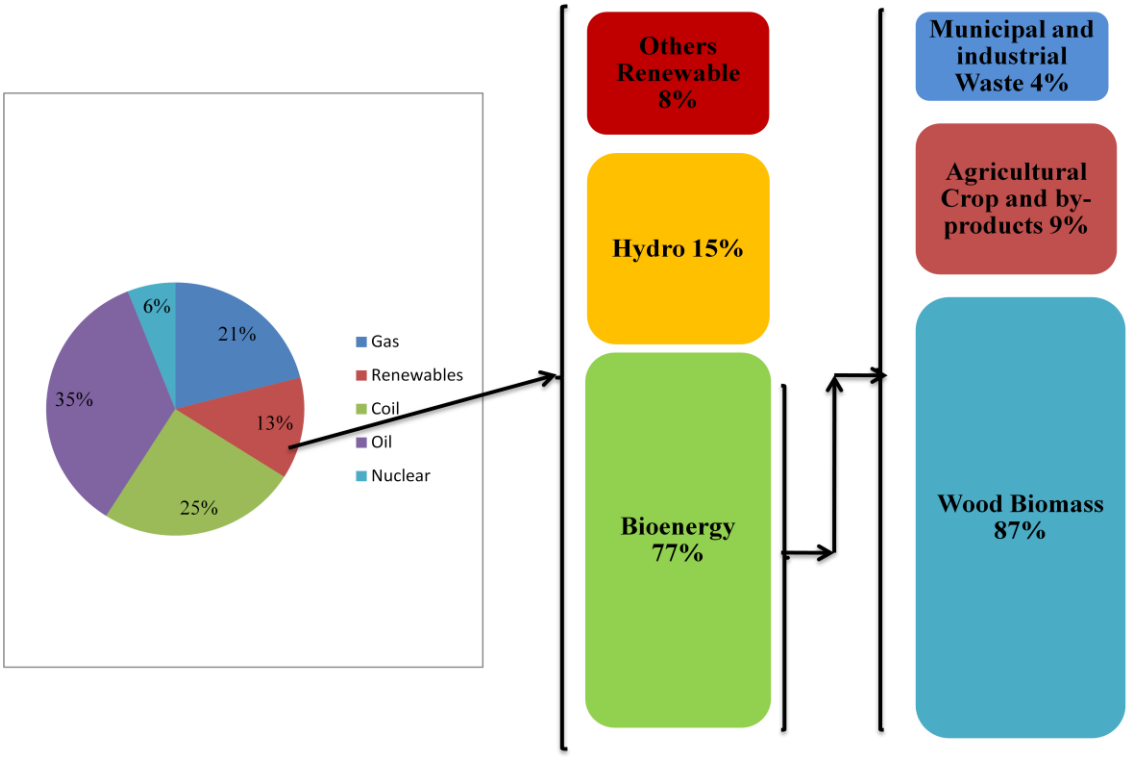

Figure 1.2 World primary energy consumption (IEA, 2009)

As biomass energy becomes one of the major global energy alternatives, many research efforts have been devoted to converting inexpensive waste biomass feedstock (forestry, municipality residues, and wastes) into bioenergy. As shown in Figure 1.3 there are many

bioenergy routes which can be used to convert raw biomass feedstock into a final energy product: Thermal and biochemical conversions.

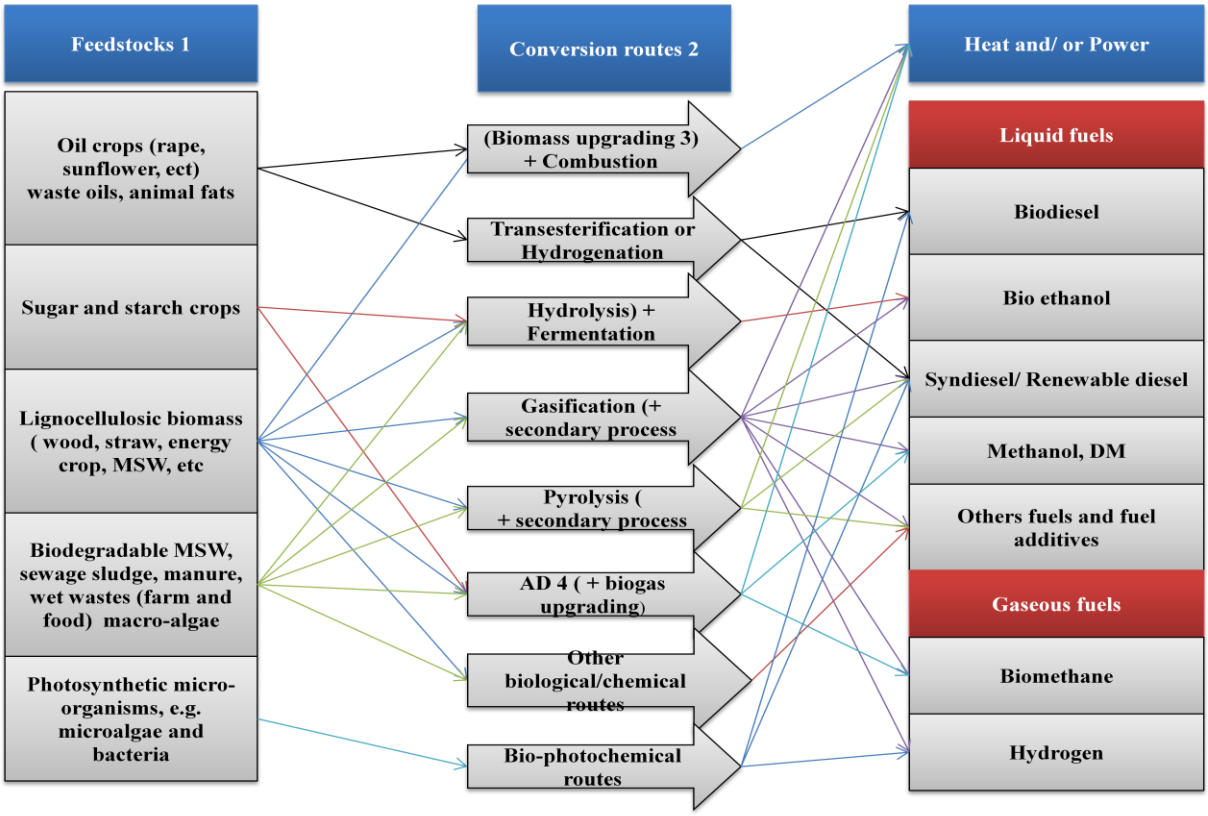

Figure 1.3 Synthetic view of the wide variety of bioenergy routes (IEA, 2009).

In the technology of thermal conversation, heat is the main mechanism to convert the biomass into other biofuels. The common methods used are combustion, pyrolysis torrefaction, hydrothermal upgrading, hydroprocessing, co-firing and gasification (which produces a mixture of hydrogen (H_2), CH_4, CO_2, carbon monoxide (CO), nitrogen (N_2) gas (Veziroglu, 1995).

Biochemical conversions are processes in which bacterial enzymes and other micro-organisms are used to perform anaerobic digestion, (fermentation) and composting into other forms of biofuels such as biogas, bioethanol, biodiesel and biohydrogen (Tsai *et al*., 2007; de Vrije *et al*., 2002; Wilkie *et al*., 2000). But biohydrogen is still considered the ultimate solution of clean and recyclable energy carrier in a long term (Kapdan and Kargi, 2006). Biomass feedstock contains a large amount of cellulosic materials, such as cellulose, hemicelluloses, and lignin. Among those three major components, cellulose and hemicelluloses are much easier to degrade biologically, thereby, being more economically viable for energy

conversion (Chandrakant and Bisaria, 1998). The conversion of biomass to ethanol is a process based on yeasts, fungi and bacteria microorganisms, which can effectively ferment 5 carbon sugar and 6 carbon sugars to ethanol. The specific yeast *saccharomyces cerevisiea* is frequently used to ferment glucose to ethanol (Rutz and Janssen 2007).

1.1.3.12 Types of biofuel from biomass

Biofuels are gaining increased public and scientific attention, driven by factors such as increasing oil prices, the need for increased energy security, concern over greenhouse gas emissions from fossil (IEA, 2007a). These biofuels are the perfect alternatives of fossil fuels. The most common types of biofuels are:

- **Biodiesel**

Biodiesel can be produced from new or used vegetable oils (soybean oil) or animal fats, which are nontoxic, biodegradable, and renewable (US DOE, 2010). The process used is chemical or enzymatic transesterification which involves alcohol (methanol) to create a catalytic reaction. The catalyst is usually a strong base, such as sodium or potassium hydroxide. The new chemical compounds would be methyl esters or biodiesel (Gerpen, 2005).

- **Bioalcohols**

There are four basic alcohols: Butanol, Ethanol, Methanol and Propanol, although bioethanol is the most common biofuel, accounting for more than 90% of total biofuel usage. Conventional production is a well-known process based on enzymatic conversion of starchy biomass into sugars, and/or fermentation of 6-carbon sugars with final distillation of ethanol to fuel grade (IEA, 2007a). Ethanol can be produced from any biological feedstock that contains appreciable amounts of materials that can be converted into sugars such as cereal crops, maize, sugar-cane, sugar beets, potatoes, sorghum, and cassava (Rutz and Janssen, 2007). The world's largest producers of bio-ethanol are Brazil (sugar-cane ethanol) and the United States (corn ethanol) (CFDC, 2007).

- **Biogas**

Biogas is a safe, clean, affordable and sustainable source of energy from anaerobic digestion of animal manures, agricultural residues, industrial wastewater, human waste and other organic materials (BBL, 2008). The result of this process is the production of CH_4 and CO_2,

which make up 2/3, and 1/3 of the total gas produced respectively. Small amounts of N_2, H_2 and hydrogen sulphides (H_2S) also occur. The gases CH_4 and H_2 can be used as a fuel for cooking, lighting, and power generation (Agama, 2007). Biogas is therefore, indeed the key to unlock a comprehensive rural economic development strategy that can contribute significantly to improved and sustainable livelihoods (BBL, 2008).

- **Solid biofuel**

The majority of the population in rural areas rely on human energy and labour for agriculture and transport. Formal energy consumption pattern is dominated by solid biofuels (90%), the most part is consumed by households including: wood residues, domestic refuse, agricultural waste, forest residues, energy crop, manures (Rutz and Janssen, 2007). The easiest way of using it is to burn it directly on a stove or furnace to provide heat or raise steam. However, when solid biofuel is in an inconvenient form, the only treatment needed is to densify it. This process includes grinding the raw biomass to an appropriate particulate size (Balat, 2006).

- **Biohydrogen**

Today, to exploit alternative energy sources has become a pressing agenda due to recent global energy concerns and geopolitical instability. To replace fossil fuels, attention is being paid to the usage of renewable bioenergy viewed as one of the ways to alleviate the current global warming crisis (Bockris, 2002). As biomass energy becomes one of the major global energy alternatives, many research efforts have been devoted to converting inexpensive waste biomass feedstock (e.g. agricultural wastes) into bioenergy, such as ethanol, biodiesel, and hydrogen (Tsai *et al*., 2007; de Vrije *et al*., 2002; Wilkie *et al*., 2000). Among the candidates of new resources, H_2 is considered an ideal energy carrier for the future (Davis and Higson, 2007; Lovely, 2006). It is not a primary energy source, but rather serves as a medium through which primary energy sources (e.g. nuclear or solar energy) can be stored, transmitted and utilized to fulfil our energy needs (Das and Veziroglu, 2001). The advantages of this sustainable energy source are numerous: it is clean, efficient, renewable, and does not generate any toxic by-product, as it can be produced by water decomposition (Hansel and Lindblad, 1998).

1.1.4 H_2 properties and applications

H_2 is the most plentiful element in the universe, making up about three-quarters of all the matter. In normal conditions, it is a colourless, odourless, insipid and harmless gas to humans and the environment. It has an enthalpy of combustion of 121 kJ/g, which is the highest per unit weight of all chemicals (Bockris, 1981; Suzuki, 1982). H_2 offers the world a stable and sustainable future. The future uses will be enhanced by the success of the H_2 fuel cell and the H_2 car. H_2-powered cars will increase the demand for H_2 production and allow the economics of H_2 power to utilise the mass market efficiencies. The possibility of converting H_2 into electricity via fuel cells makes the application of H_2 energy very promising (Moore and Raman, 1998). As Oxygen (O_2) scavenger, H_2 is also used to remove trace amount of O_2 to prevent oxidation and corrosion, in methanol production, in hydrodealkylation, hydro-cracking, and hydrodesulphurization. Finally, as a coolant in electrical generators.

The major advantages of H_2 are its ability to be stored compactly as a metallic hydride and the production of water as the only product resulting from its combustion (Reaction 1.1) (Billings, 1991). $H_2 + ½O_2 \rightarrow H_2O + 286$ kJ (Reaction 1.1) (Da Rosa, 2005).

1.1.5 Biohydrogen as renewable energy

H_2 can be generated in 4 ways: (1) electrochemical processes; (2) thermochemical processes; (3) photochemical process, photocatalytic process, or photo electrochemical process and (4) Fermentative hydrogen production. The first 3 processes have the disadvantages in that they do not reduce waste, do not produce energy, but consume it through the use of electricity derived from fossil fuel combustion. On the other hand, fermentative H_2 production produces energy and reduces waste (Han and Shin, 2004). Fermentative H_2 production can be classified into two categories: anaerobic fermentation and photosynthesis (Kotsopoulos *et al*., 2006). The efficiency of photosynthetic H_2 production is low and cannot be operated in the absence of light. Furthermore, photosynthetic H_2 production rates are relatively low, from 0.07 to 0.16 mmol of H_2/ (L/h) (Levin *et al*., 2004). In contrast, fermentative H_2 can produce H_2 all day without light, using various kinds of substrates such as organic wastes, and has higher H_2 production rate reaching 120 mmol of H_2/(L/h), simple control requirements, lower operating costs and higher feasibility for industrialization (Levin *et al*., 2004; Li and Fang,

2007; Yokoi *et al*., 1995). Thus, fermentative H_2 production is more feasible and widely used. It is of great significance to produce H_2 from organic wastes by fermentative H_2 production, because it plays the dual role of waste reduction and energy production (Wang and Wan, 2008a; Xing *et al*., 2008).

1.1.6 Biological hydrogen production processes

Biological H_2 production processes can be classified as follows (Table 1.1): Bio-photolysis of water using algae and cyanobacteria (Direct and indirect biophotolysis), Photofermentation, Dark fermentation, Hybrid systems (Using dark-fermentative and photo-fermentative bioreactors and bio-electrochemically assisted microbial bioreactors (Das *et al*., 2008).

Table 1.1 Overview of currently known biological hydrogen production process (Benemann, 1996)**.**

Process	General Reaction	Microorganisms
1 Direct Bio-photolysis	$2H_2$ + Light→$2H_2$+O_2	Microalgae
2. Photo-fermentations	$CH_3COOH + 2H_2O$ + Light→ 4 H_2 + 2 CO_2	Purple bacteria, Microalgae
3. Indirect bio photolysis	a) $6H_2O + 6\ CO_2$ + Light → $C6H_{12}O_6 + 6O_2$ b) $C_6H_{12}O_6 + 2H_2O$ → 4 H_2 + 2 CH_3COOH +2 CO_2 c) 2 CH_3COOH + 4 H_2O + Light → 8 H_2 + 4 CO_2 Overall reaction: 12 H_2O + Light → 12 H_2 + $6O_2$	Microalgae, Cyanobacteria
4Water Gas Shift Reaction	CO + H_2O→ CO_2+ H_2	Fermentative bacteria, Photosynthetic bacteria
5 Two-Phase H_2+CH_4 Fermentations	a) $C_6H_{12}O_6 + 2\ H_2O$ → 4 H_2 + 2 CH_3COOH +2CO_2 b) 2 CH_3COOH→ 2 CH_4 + 2 CO_2	Fermentative bacteria Methanogenic bacteria
6 High-Yield Dark Fermentations	$CH_{12}O_6 + 6H_2O$ → 12 H_2 + 6 CO_2	Fermentative bacteria

1.1.7 Microbiology of biohydrogen production

The critical issue in biohydrogen production is use of effective microbes. Strict and facultative anaerobes (*Clostridia*, *Micrococci*, *Methanobacteria*, *Enterobacteria*, etc), aerobes (*Alcaligenes* and *Bacillus*) and photosynthetic bacteria are often used for H_2 generation (Davila-Vazquez *et al*., 2008; Levin *et al* 2006; Wang and Wan, 2009; Yokoi *et al*, 2002). The anaerobic H_2 fermentation processes from *Clostridium* sp and *Enterobacter* are the most well studied (Fang *et al*., 2002; Wu *et al*., 2006). Species of genus *Clostridium* are gram-positive, rod-shape, strict anaerobes and endospore formers, which develop when the environmental conditions become unfavourable (e.g. high temperature, desiccation, carbon or nitrogen deficiency, chemical toxicity). When favourable conditions return, the

spores germinate and become vegetative cells whereas *Enterobacter* are gram- negative, rod-shaped, and facultative anaerobes meaning it is capable of growing in aerobic and anaerobic environments. As a facultative anaerobe it is able to ferment glucose and lactose and create H_2 gas through these metabolic processes (Wang and Wan, 2009). The production cost of hydrogen could limit their widespread application as an energy source in the future. The bacteria can be obtained in natural environments such as soil, wastewater sludge and compost etc. (Wang and Wan, 2009). These materials can be used as inoculums for fermentative H_2 production. Inocula are selected either as pure or mixed cultures. When mixed cultures are involved in the anaerobic pretreatment process, an enrichment procedure for production of suitable inoculums is necessary for biohydrogen production. In most cases, acid, base, aeration, freezing and thawing, chloroform, short hydraulic retention time and heat-shock pretreatment methods have been used to suppress as much H_2 producing microorganisms as possible and to preserve the activity of H_2 consuming bacteria (Eun *et al*., 2004; Setlow, 2003). Heat-shock has been the most pretreatment method used. But it is not always effective to kill H_2 consuming microorganisms, for it may inhibit the activity of some bacteria (endospore bacteria such as *Clostridium*) (Kraemer and Bagley (2007).

In all previous studies, H_2 was produced by acidogens grown in suspension. However, it was demonstrated in many studies that the H_2 producing biomass can also develop into granules with high bioactivity (Herbert *et al*., 2001). Besides fermentation types, H_2 production capability is also directly related to fermentation substrates. Each substrate has its own fermentation pathways and end-products, which leads to different H_2 yields (Aoyama *et al*., 1997; Taguchi *et al*., 1995; Wang *et al*., 2008b; Woodward *et al*., 1996). Several monosaccharides, including hexoses and pentoses, have been often used as fermentation substrates. They are the hydrolysate of a wide variety of macromolecules, such as starch, cellulose and hemicellulose, and are abundantly available in various industries.

1.1.8 Substrates

The main criteria for substrate selection are: availability, cost, carbohydrate content and biodegradability (Kapdan and Kargi 2006). Glucose, sucrose, starch and cellulose, have been extensively studied as carbon substrates for biohydrogen production. They have been used as model substrates for research purposes due to their easy biodegradability. Most of them can be present in different carbohydrate-rich wastewaters and agricultural wastes. Other substrates suitable for biohydrogen production are protein- and fat-rich wastes, less available

than carbohydrate (Svensson and Karlsson 2005). A maximum theoretical yield of 12 mol of H_2 per mol of hexose is predicted from the complete conversion of glucose (Reaction 1.2).

$C_6H_{12}O_6 + 6H_2O \rightarrow 12\ H_2 + 6CO_2\ \Delta G°' = -25$ kJ/mol. Reaction 1.2

Cellulose which is the principal component of plant biomass is the most abundant renewable natural resource in the world and is a promising economical source for H_2 production (Haug, 1993; Weimer and Zeikus, 1977). The conversion of cellulose to H_2 by microbial fermentation, therefore, represents a partial answer to waste accumulation, the depletion of hydrocarbon fuel reserves, and carbon-dioxide release (Ueno *et al*., 2001a; Zeikus, 1980). However, its hydrolysation is very difficult, which has limited its uses (Lynd *et al*., 2002). The addition of exogenous cellulose enzymes is necessary for hydrolysis of cellulose to generate H_2 by mesophilic anaerobic bacteria (Datar *et al*., 2007). Thermophilic anaerobic bacteria can effectively utilise cellulose (Lynd *et al*., 1989) and therefore, they have great potential for H_2 production from cellulose without the addition of exogenous cellulase. In general, thermophiles are thought to be robust microorganisms that contain stable enzymes. It is reported that more H_2 is produced under thermophilic conditions than under mesophilic conditions (Zhang *et al*,.2003) and thermophilic anaerobes such as *Thermoanaerobacterium, thermosaccharolyticum* have nearly equivalent H_2 production yields compared to *Clostridium butyricum* (Ueno *et al*,. 2001a).

Chapter 2
Production of Hydrogen by Dark fermentation: Mechanisms for reductant disposal

2.1 Dark fermentation

Among the H_2 production methods, the most promising and environmentally friendly method seems to be dark fermentation from organic wastes as it combines the H_2 generation with waste treatment (Benemann, 1996). Dark fermentation is a conversion of biomass to H_2, CO_2 and/or CH_4, and other products, such as acids (lactic ($C_3H_6O_3$), acetic (CH_3COOH), butyric ($CH_3CH_2CH_2COOH$), propionic (CH_3CH_2COOH) formic acid (HCOOH) etc) and alcohol (ethanol (C_2H_5OH), butanol (C_4H_9OH), propanol ($CH_3CH_2CH_2OH$) builds on anaerobic digestion of carbohydrate-rich substances under influence of bacteria (Urbaniec and Grabarczyk, 2009). The decomposition of biowaste occurs in four stages as shown in Figure 2.1: hydrolysis, acidogenesis, acetogenesis and methanogenesis.

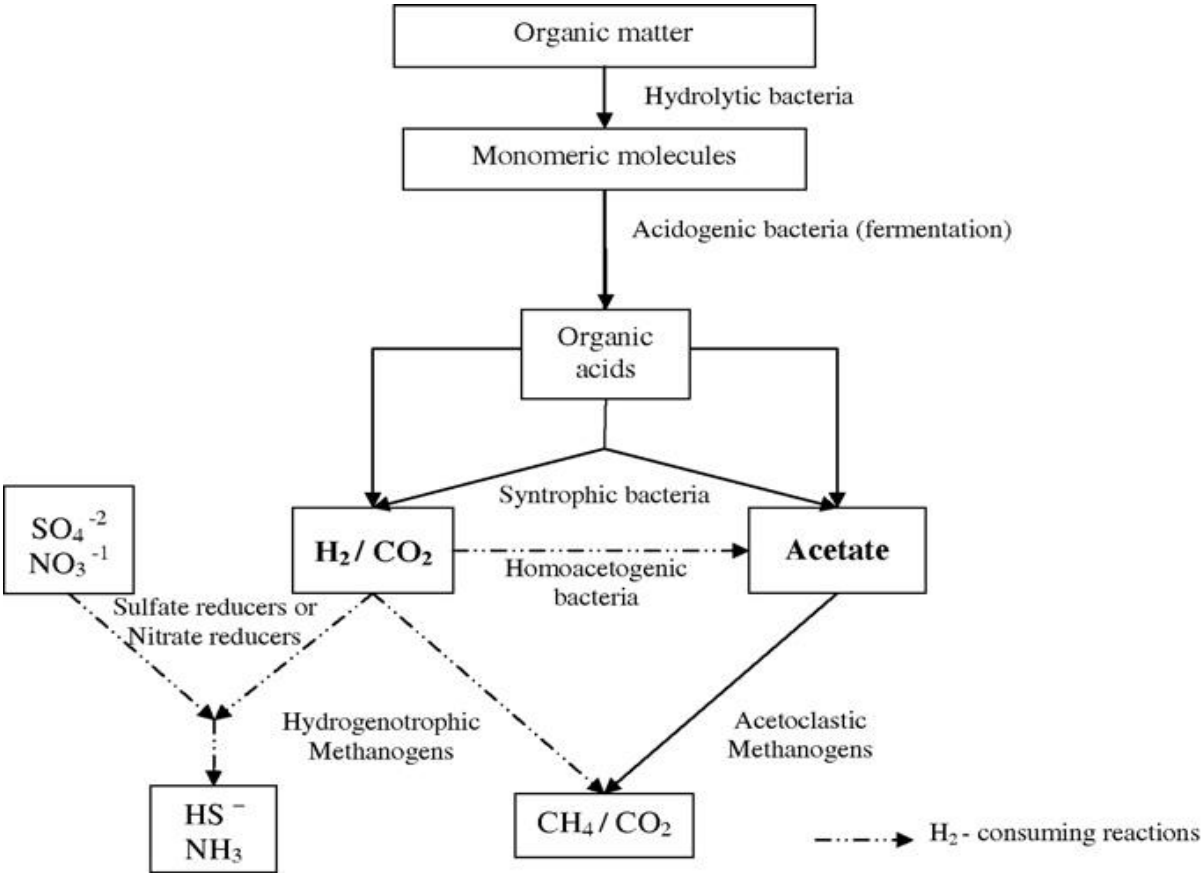

Figure 2.1 Degradation steps of anaerobic digestion process (Valdez-Vazquez and Poggi-Varaldo, 2009)

2.1.1 Hydrolysis

During this phase, enzymes: lipases, proteases, cellulases, amylases, etc. produced by hydrolytic bacteria transform the insoluble complex organic matter such as carbohydrates, cellulose, proteins and fats into soluble liquefied monomers and polymers (amino acids, fatty acids, and simple sugars) (Reaction 2.1) (Ostrem, 2004).

$C_6H_{10}O_4 + 2H_2O \rightarrow C_6H_{12}O_6 + 2H_2$ Reaction 2.1:

2.1.2 Acidogenesis

Acidogenesis is the next step of anaerobic digestion in which the hydrolyzed compounds are fermented into volatile fatty acids (VFAs), alcohols, and biogas (Reith *et al*., 2003). The principal acidogenesis stage products are: CH_3CH_2COOH, $CH_3CH_2CH_2COOH$, CH_3COOH, HCOOH, $C_3H_6O_3$, C_2H_5OH) and methanol (CH_3OH) (Ostrem, 2004; Bilitewski *et al*., 1997) . This phase 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 (Gavrilescu, 2002). H_2, CO_2 and CH_3COOH will skip the third stage, acetogenesis, and be utilized directly by the methanogenic bacteria in the final stage (Bilitewski *et al*., 1997; Ostrem, 2004). Typical reactions in the acid-forming stages are shown below where glucose is converted to ethanol, (Reaction 2.2), to propionate (Reaction 2.3) and to acetic acid (Reaction 2.4).

$C_6H_{12}O_6 \leftrightarrow 2CH_3CH_2OH + 2CO_2$ Reaction 2.2

$C_6H_{12}O_6 + 2H_2 \leftrightarrow 2CH_3CH_2COOH + 2H_2O$ Reaction 2.3

$C_6H_{12}O_6 \rightarrow 3CH_3COOH$ Reaction 2.4

2.1.3 Acetogenesis

The third step, known as acetogenesis is part of the fermentation process where the rest of the simple molecules from Acidogenesis are further digested to produce hydrogen, carbon dioxide and acetic acid. In this process, the reaction will occur if the H_2 partial pressure is low enough to thermodynamically allow the conversion of all the VFAs (Mata-Alvarez, 2003). Reaction 2.5 shows the conversion of propionate to acetate, only achievable at low hydrogen pressure (Ostrem, 2004).

$CH_3CH_2COO^- + 3H_2O \leftrightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2$ Reaction 2.5

2.1.4 Methanogenesis

Methanogenesis constitutes the final step of anaerobic digestion in which bacteria convert H_2, CH_3COO^- and CO_2 and CH_3OH to CH_4. Among these, CH_3COO^- is the most important, since about 60% of the CH_4 produced is derived from acetate and only (40%) from CO_2 (Evans, 2001). The bacteria responsible for this conversion are called methanogens and are strict anaerobes. There are two groups of methanogenic microorganisms: acetoclastic (Reaction 2.6) methanogenesis and hydrogenotrophic methanogenesis (Reaction 2.7).

Acetate + $H_2O \rightarrow CH_4 + HCO^-_3$ ΔG^o = -31 kJ/mol methane Reaction 2.6

$4H_2 + HCO^-_3 + H+ \rightarrow CH_4 + 2H_2O$ ΔG^o = -135.6 kJ/mol methane Reaction 2.7

2.2 Parameters influencing dark fermentative hydrogen production

Biological production of hydrogen, using microorganisms, is an exciting new area of technology development that offers the potential production of usable H_2 from a variety of renewable resources. It has been reported by a great number of studies that temperature, hydraulic retention time (HRT), pH, H_2 partial pressure, reactor type, VFAs and inorganic content are the main parameters that affect the anaerobic H_2 fermentation process (Das and Verizoglu, 2001, 2008; Fang *et al*., 2002; Hawkes *et al*., 2002, 2007; Kapdan and Kargi, 2006; Kraemer and Bagley, 2007; Tanisho and Kamiya, 1989).

2.2.1 H_2 partial pressures

H_2 partial pressure in the liquid phase is one of the key factors affecting H_2 production. Actual dissolved H_2 concentrations have been found to be as much as 60 fold higher than the predicted theoretical equilibrium values derived from the head space H_2 partial pressures using Henry's law (Kuroda *et al*., 1991). The quantity H_2 referred to as the actual dissolved H_2, may in fact consist of two components: solubilised H_2 and non-solubilised H_2. Under steady-state conditions, the difference between the actual dissolved H_2 concentrations in an anaerobic digester relative to the predicted thermodynamic equilibrium concentration has been estimated from the following relationship (Equation 2.1) (Pauss *et al*., 1990):

$$\frac{H_2^L}{H_2^{L*}} = \frac{HP_L}{K_H^T RTk_L} + 1 \qquad \text{Equation 2.1}$$

Where, $H_2{}^L$ (mol) is the dissolved H_2 in the bioreactor liquid phase, $H_2{}^{L*}$ (mol) is the thermodynamic equilibrium dissolved H_2 concentration, HP_L (h^{-1}) is the volumetric hydrogen productivity, $K_H{}^T$ (mol Pa^{-1}) is Henry's constant, k_L (h^{-1}) is the H_2 volumetric mass transfer coefficient, R (8.314 Pa $mol^{-1}K^{-1}$) is the ideal gas constant, and T (K) is temperature. Under ideal conditions, the thermodynamic equilibrium concentration of dissolved H_2 is related to partial pressure by Henry's law as follows (Equation 2.2):

$$p = K_H^{T^o} H_2^{L*} \qquad \text{Equation 2.2}$$

Where p is the partial pressure of H_2, K_H^T equals 1282.05 L.atm/mol at T^o = 298 K. The production of liquid phase entrapped H_2 within the bioreactor bed and its subsequent transfer to the vapour phase within the gas disengager is described by these two equations 2.3 and 2.4:

$$V_L \frac{dH_2^L}{dt} = HP_L - k_L A\left(H_2^L - \frac{H_2^V}{K_H^T}\right) \qquad \text{Equation 2.3}$$

$$V_V \frac{dH_2^V}{dt} = k_L A\left(H_2^L - \frac{H_2^V}{K_H^T}\right) - H_2^V Q_V \qquad \text{Equation 2.4}$$

Where V_L (L) is the volume of the bioreactor's liquid phase, V_V (L) is the volume of the bioreactor's liquid-free vapour phase, H_2^V is the partial pressure of H_2 in the bioreactor's vapour phase, Q_V is the gas or vapour flux from the bioreactor, A (m^2) is the gas exchange surface area. The volumetric mass transfer coefficient k_L is the rate limiting constant for H_2 disengagement from the liquid phase. Its value depends on H_2 diffusivity, effluent recycle rate, temperature, cavitations or bubble formation rate, and liquid viscosity (Equation 2.5):

$$K_H^T = K_H^{T^o} e^{\left[-C\left(\frac{1}{T} - \frac{1}{T^o}\right)\right]} \qquad \text{Equation 2.5}$$

Where C equals 500 K for H_2 and T^o = 298 K.

2.2.2 Effect of temperature

Temperature is one of the most relevant parameter that affects the activity of H_2 producing bacteria by influencing the activity of some essential enzymes such as hydrogenases for biological hydrogen fermentation (Wang and Wan, 2008b). Most biohydrogen production processes can be operated at different temperature: mesophilic (25°C-40°C), thermophilic (40°C-60°C), and extreme thermophilic (65°C-80°C) or hypethermophilic (>80°C) (Davila-Vasquez *et al*., 2008). Among them, mesophilic bacteria are the most extensively studied. It has been reported that thermophilic temperature provide a number of advantages compared with the mesophilic. Firstly, thermophilic bacteria can achieve the theoretical yield of 4 mole H_2 per mole glucose, where the ones at mesophilic conditions were normally less than 2 mole H_2 per mole glucose (Van Niel *et al*., 2004). This is possible through acetate end product reaction, decreasing or preventing butyrate, ethanol and propionate product reaction (Kadar *et al*., 2004; Van Niel *et al*., 2004). Secondly, it has much better pathogenic destruction for

digested residues performed at high temperatures (Sahlstrom, 2003). Thirdly, it minimises the contamination by H_2 consumers such as methanogens, solventogens. The effect of temperature on volumetric H_2 production rate 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 (Valdez-Vazquez *et al*., 2005) (Reaction 2.8).

$C6H12O6 + 2H2O + 2CH3COOH + 4H2 + 2CO2$ $\Delta G° = -176.1$ kJ/mol, $\Delta H = +90.69$ kJ/mol

Reaction 2.8

Based on the changes in the Gibbs free energy and enthalpy of the reaction there are indications that the reaction can occur spontaneously and that it is endothermic. Therefore, by changing the temperature of glucose fermentation, and maintaining the concentrations of the reactants constant would enhance H_2 formation. (Sommer *et al*., 2003). The Van't Hoff equations 2.6 and 2.7 can be used to explain the effect of the temperature on the equilibrium constant (Smith *et al*., 2000).

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

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

2.2.3 Effect of pH

The influence of pH has been recognised as a key factor in determining the outcome of H_2 fermentation. The pH is related to three important facts: (a) methanogen growth limitation, (b) H_2 production performance, and (c) regulation of shift to solventogenesis (Fabiano and Perego, 2002). The methanogenesis is the critical stage for the anaerobic bio-hydrogen production process due to the rapid H_2 consumption rate of H_2 utilizing methanogens. The operation temperature and pH of H_2 production is selected as the target operation parameter because it has a significant influence on the hydrogenases activity and on the metabolism pathways of microorganisms in the mixed consortium, e.g. utilization of carbon and energy sources, efficiency of substrate degradation, synthesis of proteins (Baily and Ollis, 1986; Lay and Lin, 2004). pH variation can affect H_2 production, microbial population shift, cell

morphology and structure and, therefore, flocculation and adhesion phenomena (Ginkel *et al*., 2001; Van Niel *et al*., 2002).

2.3 Anaerobic bioreactor revolution

Recently, significant advances have been made in the development of fluidized granular bed bioreactors that have high rates of biohydrogen generation (Zhang *et al*., 2008a). Maintenance of high biomass densities under high dilution rates without significant biomass loss through washout, is a major advantage that granular bed bioreactors have over most of the previous anaerobic bioreactor options of such fixed bed bioreactors or trickling bed bioreactors. All the recent fluidized granular bed bioreactors including continuous stirred tank reactor (CSTR), carrier-induced granular sludge bed reactor (CIGSB), anaerobic fluidized granular bed bioreactor (AFGB) and granule-based continuous stirred tank reactor (CSTR) are in reality modified versions of the classical upflow anaerobic sludge bed (UASB) bioreactors (Stronach *et al*., 1986). Many of the classical UASB bioreactors are really granulated sludge bioreactors in which a large proportion of the bacterial biomass occurs in the form of bacterial granules rather than as friable flocs or fine sludge. Usually, the granules in the classical UASB systems would have taken several months to form. Hence the typical start-up times for classical UASB systems have usually been inordinately long. A recent major advance in granulation biotechnology has been the capacity to initiate rapid induction, growth and development of bacteria granules. This advance was first made in 2004 (Lee *et al*., 2004). 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, 2008b, 2008c). In spite of the superiority of granule based bioreactor systems, the diffusion of biohydrogen biotechnology has proved to be slow. Publication of the dramatic increases in H_2 Productivities (HPs) (7.3 – 9.3 L H_2/(L.h), (Lee *et al*., 2006) that have been achieved with bacterial granular bed bioreactors appears to have had minimum impact on the broader biohydrogen bioreactor development research agenda. In this study, the bioreactor system selected was the fluidized granular bed-based bioreactor system. This system offers a number of degrees of freedom with respect to process operation variables such as HRT and effluent recycling. The granular bed can be conceptualised as a quasi-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 (e.g. H_2 and CO_2) between the mobile bulk phase and the fluidized stationary granular phase.

2.3.1 Bacterial granulation

There are two main forms of granulation which are aerobic and anaerobic granulation. Anaerobic are extensively studied and are considered a major reason of the successful introduction of the UASB reactor concept for anaerobic treatment of industrial effluents (Hulshoff Pol *et al*., 2004). As a result of drawbacks of anaerobic granulation technology (e.g. long start-up period, a relatively high operation temperature, unsuitability for low-strength organic wastewater treatment especially for removal of nutrients Nitrogen and Phosphorous from wastewater), aerobic granulation has been a centre of interest and has since been reported in sequencing batch reactors (SBRs) despite its infancy. This technology has numerous applications in high-strength organic wastewater treatment, simultaneous nitrogen, organic and phosphorus removal, phenolic wastewater treatment and biosorption of heavy metals (Liu and Tay, 2004). Understanding the granulation process is highly relevant for improving reactor design and performance (Tay *et al*., 2006). Thus, to achieve this purpose, the mechanisms of granulation should be fully understood.

2.3.1.1 Mechanism of granular sludge

It is clear from the mechanism that several conditions need to be fulfilled in order for aerobic and anaerobic granules to form. No individual stage can explain the granulation process. Thus, it is a combination of numerous factors: Physical, Chemical and biological that allow for granulation to proceed (Liu *et al*., 2003).

1. Induction of physical forces to promote initial bacterial contact, with each other or to growth nuclei. The forces would include, hydrodynamic, diffusion, gravity, thermodynamic and cell mobility forces.

2. Physico-chemical forces of attraction to stabilise microcolony growth, the forces involved: Gibbs energy, (van der Waals).

3. Surface tension, opposition charge attraction, hydrophobicity, and interaction of filamentous bacteria with other cells.

4 Biochemical forces including cell surface dehydration membrane, membrane fusion, quorum sensing and signalling systems with the microbial community.

5 Interactions amongst bacterial communities with influencing factors like extracellular products (ECP) and alteration of metabolic pathways as a result of organisation within a biofilm.

6 Formation of the three-dimensional microbial aggregate which is shaped by hydrodynamic shear forces within the bioreactor. This removes any loosely attached bacteria and forms a highly structured, spherical micro community in granules. The overall shape and structure thereof, is determined by bacterial interactions and species, shear forces and substrate loading rate into the reactor.

2.3.1.2 Factors influencing granulation formation

In order to optimise and enhance bacterial granulation for industrial bioprocessing applications, various conditions must be taken into consideration such as temperature, pH, HRT, substrate loading rates (OLR), inorganic nutrient supplementation for enhancement of biohydrogen production type substrate and fluidization of the bacterial cell within the reactor (Tay *et al*., 2006).

2.3.1.3 Carrier matrix for cell immobilisation

The matrix used for cell immobilisation have become common alternatives to suspended-cell systems in continuous operations since they are more efficient in solid/liquid separation and can be operated at high dilution rates (or low retention times) without encountering washout of cells (Chang *et al*., 2002). Generally, the carriers used for immobilising cells are porous materials (loofah sponge), activated carbon, sand, and several other substances. It was found that activated carbon seems to be a better choice since it allowed higher biomass yield, exhibition H_2 generation rate and also showed more stability when operated at low hydraulic retention rate (Chang *et al*., 2002).

In an AFGB biofilm, activated carbon carriers are known to occupy a volume between 30% - 40% of the total volume of the reactor column (at the bottom of the up flow reactor) (Thompson, 2005). It has been reported that the activated carbon carriers provide an additional surface area for attached-growth bacteria, which increases the density of the resultant biomass, with concomitant improved settling (Hulshoff Pol *et al*., 2004). The presence of activated carbon carriers offers three advantages: Firstly, it is shelter to ecological niches that enhance biological attachment secondly it is contributes to the development of an attached biofilm and, thirdly it is acts as a nucleus for granule formation (Hulshoff Pol *et al*., 2004).

2.4 Current state of hydrogen production

With regard to the current state of biohydrogen production, the capacity to achieve high volumetric hydrogen productivities (2 900 L H_2 /m^3/h) and high hydrogen yields (> 4 H_2 /mol glucose) remains a technological challenge? A 5 kW hydrogen fuel cell which has the capacity to power a house requires a fuel supply rate not less than 2 900 L/h (Levin et al 2004). Expressed on a volumetric basis this H_2 rate is equivalent to 120 mol H_2/m^3/h. This value could be used as the minimum HP benchmark or threshold for evaluating the economic viability of a given biohydrogen generation process. For a hydrogen generation process to be commercially viable the bioreactor unit volume required to perform useful work on a 5 kW fuel cell should not be greater 1 m^3. Therefore the HP for a hydrogen bioprocess should not be less than 120 mol H_2/m^3/h. The other objective for a hydrogen bioprocess involves increasing the hydrogen yield. To increase the commercially viability of hydrogen generation the hydrogen yield of the process also needs to be maximized, possibly to greater than 4 mol H_2/mol glucose.

2.5 Problem statement

The issues confronting hydrogen production can be summarised as follows:

1. Achievement of HYs equal or greater than 3.0 mol H_2/mol glucose in bioreactor experiments have always been dependent on the following operational conditions: monocultures, thermophilic temperatures, low substrate loading rates, low dilution rates, H_2 gas stripping by sparging with N_2, maintenance of low H_2 partial pressures (< 100 Pa) and low bacterial biomass densities (de Vrije, *et al*., 2007; Zeiden and van Niel, 2010).

2. Achievement of high Hydrogen Productivities depends on the following operational conditions: undefined multispecies bacterial consortia, high substrate loading rates, high H_2 partial pressures, high dilution rates, and high bacterial biomass densities (Lee, *et al*., 2006; Zhang, *et al*., 2008a; Ngoma, *et al*., 2011). High volumetric hydrogen productivities are usually correlated with high H_2 partial pressures within the bioreactor system (Ngoma *et al*., 2011).

3. High H_2 partial pressures (>100 Pa) within the bioreactor system are usually correlated with low Hydrogen Yields, that is, Hydrogen Yields below 3 mol H_2/mol glucose.

4. Low Hydrogen Yields and high H_2 partial pressures are associated with the accumulation of the volatile fatty acids (VFAs) acetate, propionate and butyrate.

2.6 Research objectives and aims

The guiding objective was to investigate what bioreactor operation factors would result in the simultaneous increase in both Hydrogen Productivities and Hydrogen Yields. These bioreactor operational factors would include the following:

1. Bacterial granulation and bacterial biomass density.
2. Temperature.
3. Influent supply rate.
4. Efficiency of effluent gas disengagement.
5. Rate of degassed effluent recycling.

Chapter 3

Effect of temperature and effluent recycle rate on hydrogen production by a sewage derived undefined bacterial culture

3.1 Introduction

Major advances have already been made in the development of bioprocesses for the efficient production of biohydrogen as a green fuel for electricity generation (Lee *et al*., 2004; Lee *et al*., 2006; O-Thong *et al*., 2008; Thompson *et al*., 2008; Zhang *et al*., 2007a, 2007b, 2008a, 2008b, 2008c). A major motivation for these developments has been the fact that biohydrogen represents not only an environmentally friendly and sustainable energy fuel, but also has the highest energy density compared to all other fuels (142 MJ/kg). In addition, when used as an energy fuel for powering fuel cells, it can be transformed into electricity with an efficiency of 55%. It has been proposed that for electricity generation, a 5 kW fuel cell would require an H_2 supply rate of 2900 L H_2 /h (Levin *et al*., 2004). This would be equivalent to a volumetric H_2 production rate of 2.9 L H_2/ (L.h) of 120 mmol H_2 / (L.h). Volumetric H_2 productivities ranging from 7.3 L H_2/(L.h) to 9.3 L H_2/(L.h) have been achieved for mesophilic fluidized bacterial granular bed bioreactors (Lee *et al*., 2004; Lee *et al*., 2006). These H_2 productivities are 2.5 to 3.2 times higher than above theoretical rates proposed for a 5 kW fuel cell. Any additional increases in H_2 productivities would translate into smaller bioreactor volumes for electricity generation and consequently, large capital cost savings. Also, any improvement in HP through process operation optimisation will also enhance the viability of biohydrogen as fuel. This study investigates the impact of temperature and degassed effluent recycling rates on biohydrogen production by an AFGB.

3.2 Materials and Methods

3.2.1 Nutrient medium

The Endo formulation (Endo *et al*., 1982) was used as the nutrient medium for inoculums preparation and for the bioreactor experiments. The medium contained 17.8 g sucrose/L and the following mineral salts (g. L^{-1}): NH_4HCO_3 6.72, $CaCl_2$ 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}g.

3.2.2 Inoculum preparation

Fresh sewage sludge was obtained from the overflow outlet of the mesophilic anaerobic digestions at the Olifantsvlei wastewater treatment works (Johannesburg). Sewage sludge samples were incubated at 90ºC for 2 hours. After the heat treatment, the pH of the sludge was reduced to pH 2.0 with 0.1 N HCl. Samples at this pH were in sealed airtight bottles for 12h at room temperature and then readjusted to pH 7.0 by mixing with Endo medium (50% v/v). Inoculum for the bioreactor experiments was prepared by incubating 500 ml sludge-Endo medium mixture in 1000 ml sealed Schott bottles overnight in a shaking incubator (100 rpm) at 65ºC. Bioreactors were inoculated with 2 L of overnight cultures.

3.2.3 Bioreactor design and set-up

The bioreactor system consisted of the following 4 components: an influent and recycle effluent inlet manifold, tubular bioreactor, tubular decanting vessel, and a tubular gas-disengager (Figure 3.1). Clear Perspex hollow tube was used for the construction of the tubular bioreactor (internal diameter (ID): 80 mm; height (H): 1000 mm). The working volume of the bioreactor's fluidized bacterial granular bed was 5L and the volumetric H_2 productivity was expressed based on this working volume rather than the total volume of the bioreactor. A 3L liquid-solid separator (ID: 140 mm and H: 200 mm) was fitted over the top end of the tubular bioreactor for solid-liquid separation to prevent the washout of the granules from bioreactor. At the base of the bioreactor, the clear Perspex cylinder was connected to a conical shaped diffuser (ID: 80 mm and H: 150 mm) made from PVC which functioned as the primary inlet for the effluent recycle stream. A stainless steel sieve (32 meshes) was fixed over the inlet of the diffuser. Above the stainless steel sieve, the conical diffuser was

filled with a 100 mm layer of 5 mm glass beads. Positioned in the middle of the diffuser were 4 inlet ports (ID 5 mm) with each inlet arranged at 90^{o} with respect to the two other inlets on each side. Nutrient medium (influent stream) was supplied directly into the upper glass bead layer via 4 inlet ports. The effluent overflow from solid-liquid separator was decanted into a gas-disengager which consisted of a gas collection cylinder (H: 200 mm and ID: 150 mm) connected to 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 between 15rpm and 50 rpm which gave a volumetric pumping rate ranging from 1.3 L/min to 3.5 L/min. The second effluent outlet drained the effluent overflow from the gas-disengager. The lid of the gas collection cycle contained a gas outlet port which was connected to a gas meter (Ritter drum-type gas meter TG 05/3). The liquid-gas separator or gas-disengager had a 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, solid-liquid separator, gas-disengager, diffuser, and piping) was 10.47 L. Bioreactor and gas-disengager temperatures were maintained at the two different operational temperatures (45ºC and 70ºC) by circulating heated water from a heated water bath through the bioreactor and gas-disengager water jackets. A Watson-Mallow Bredel (model 520U) peristaltic pump (Falmouth, UK) was used to pump the Endo nutrient into the bioreactor.

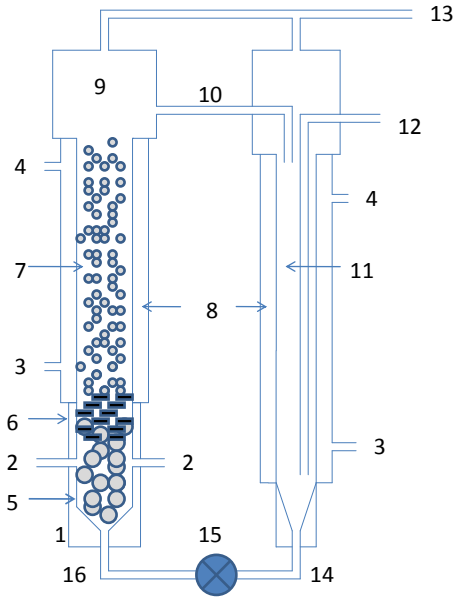

Figure 3.1 Bioreactor prototype for the simultaneous achievement of high HPs and HYs. Diagram labels: 1 – inlets manifold; 2 – influent inlets; 3 – water jacket inlets for heat exchanger; 4 – water jacket outlets for heat exchanger; 5 – bed of glass bed (5 mm) in effluent/influent diffusion and cavitation 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; effluent recycle outlet pipe; 14 effluent gas disengagement, 15 – effluent recycle pump; 16 – effluent recycle inlets.

3.2.4 Bacterial granule induction

On top of the glass bead bed, a 100 mm bed of cylindrical activated carbon (CAC) particles (diameter = 2.5 mm and length = 5.0 mm) was used to facilitate the induction of bacterial granulation in the bioreactor (Lee *et al*., 2004) see (Figure 3.2). Prior to its use, the CAC was first washed with distilled water to remove all the suspended fine particles and then sterilized by autoclaving for 20 minutes. Sufficient CAC was added to the bioreactor to give a settled bed height of 100 mm. Endo medium (3.0 L) and seed inoculum (2.0 L) which consisted of an overnight culture was added to the bioreactor system. Following inoculation, the bioreactor was operated on a batch effluent-recycle mode for 48 h at 45^{o}C to acclimatise the bacteria and allow for their attachment to the CAC. After this acclimatisation period, the bioreactor operation was switched to continuous effluent recycle mode with an initial HRT of 8 h. The HRT was then gradually decreased over 2 day intervals by increasing the nutrient medium supply rate. As the HRT was decreased from 8 to 4 h, the growth and development

of bacterial biofilm on the CAC particles became visible. With further decreases in the HRT below 4h, the biofilm growth increased and bacterial granules began to form and accumulate at the surface of the expanded CAC bed. Once granule formation had been initiated, further reductions in the HRT between 2 and 1.6 h resulted in the rapid growth and expansion of the granular bed. Granule induction, initial growth and initial development were carried out at 45^{o}C. For granule induction, the bioreactor was inoculated with 2 L of inoculums and then incubated in batch mode on Endo medium for 24 h. After 24 h, the bioreactor was supplied continuously with Endo medium starting at 15 ml/min for 24 h.

Figure 3.2 (A) Image of the AFGB column with CAC coated with bacterial flocs (biofilm) (B) shows the full bed granulated AFGB during fermentative biohydrogen production.

3.2.5 Analytical techniques

Gas chromatography was used to analyse % of 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 minute^{-1}. Sample gas injection volume was 40 µl. The following formula (equation 3.1) was used for converting total bioreactor gas flux (L/h) to mmol H_2/h.

$$\frac{\Delta H_2}{\Delta t} = \frac{P\left[\left(\%H^{GC}\right)\frac{\Delta V}{\Delta t}\right]}{RT}. \qquad \text{Equation 3.1}$$

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.

3.2.5 1 Sucrose analysis

The concentration of sucrose in the reactor effluent and feed was measured calorimetrically 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 of H_2O. Thereafter, sucrose standard curves were then generated by mixing a known dilution of this standard (sucrose standard solution) with H_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 at 80^oC 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 the method described above.

3.2.5 2 Chemical oxygen demand (COD)

A colorimetric assay was performed to determine chemical oxygen demand of influent and effluent streams. The digestion solution contained 2.6 g potassium dichromate ($K_2Cr_2O_7$) and 8.33 g mercuric sulphate ($HgSO_4$) dissolved in 42 ml 95-99 % H_2SO_4, to which 208 ml dH_2O was added. The catalyst solution consisted of 5.06 g silver sulphate (Ag_2SO_4) added to 500 ml 95-99 % H_2SO_4. Potassium hydrogen phthalate (PHP) was used as the COD standard for the standard curve. Standard curve stock solution was prepared by dissolving 765 mg PHP into 1 L dH_2O which was equivalent to 900 mg COD/L. From the stock solution, the following COD standards were prepared (mg COD/L): 50; 100; 250; 500 and 750. For COD analysis, 2.0 ml of the standard solutions were dispensed into 10 ml digestion tubes containing 1.5 ml digestion solution and 3.5 ml catalyst solution. After sealing the digestions tubes with a screw on top, the tubes were placed into a heating block (HI839800 COD Reactor, Hanna Instruments) for two hours at 150°C. After cooling, absorbance was measured at 600 nm. A similar procedure was followed for the bioreactor influent and

effluent samples. COD analysis was also used to verify the accuracy of the sucrose-resorcinol colorimetric analysis of the influent and effluent sucrose concentrations.

3.2.6 Effluents recycle rate and effluent gas disengagement

In anaerobic digesters, actual dissolved H_2 concentrations have been found to be as much as 60 fold higher than the predicted theoretical equilibrium values derived from the head space H_2 partial pressures using Henry's law (Kuroda *et al*., 1991). The quantity H_2 referred to as the actual dissolved H_2 may in fact consist of two components: solubilised H_2 and non-solubilised H_2. Non-solubilised 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-solubilised H_2 would be undergoing rapid dynamic reversible exchanges with solubilised H_2 resulting in a super-saturated equilibrium concentration of soluble H_2 in the liquid phase within the digester or bioreactor. Under steady-state conditions, the difference between the actual dissolved H_2 concentrations in an anaerobic digester relative to the predicted thermodynamic equilibrium concentration has been estimated from the following relationship (Equation 3.2):

$$\frac{H_2^L}{H_2^{L*}} = \frac{HP_L}{K_H^T RTk_L} + 1 \qquad \text{Equation 3.2}$$

Where, $H_2{}^L$ (mol) is the supersaturated concentration of dissolved hydrogen in the bioreactor liquid phase, $H_2{}^{L*}$ (mol) is the thermodynamic equilibrium dissolved H_2 concentration, HP_L (h^{-1}) is the volumetric hydrogen productivity, $K_H{}^T$ (mol Pa^{-1}) is Henry's constant, k_L (h^{-1}) is the H_2 volumetric mass transfer coefficient, R (8.314 Pa $mol^{-1}K^{-1}$) is the ideal gas constant, and T (K) is temperature. Under ideal conditions, the thermodynamic equilibrium concentration of dissolved H_2 is related to partial pressure by Henry's law as follows:

$$p = K_H^{T^o} H_2^{L*} \qquad \text{Equation 3.3}$$

Where p is the partial pressure of H_2, $K_H{}^T$ equals 1282.05 L.atm/mol at T^o = 298 K. The goal of the effluent gas disengager in this study was to reduce to the concentration of H_2 trapped in the effluent to its thermodynamic equilibrium concentration. This was accomplished by facilitating the maximum transfer or release of H_2 from the liquid phase within the gas disengager to the vapour phase, which in turn was continuously exhausted from the gas disengager. High rates of effluent recycling between the bioreactor and the gas disengager generated a high degree of fluid turbulence and cavitations within the gas disengage tube.

This process facilitated the release of super saturated dissolved H_2 through bubble production. Efficient removal of H_2 trapped in the effluent phase by the gas disengagement would increase the efficiency of the bioreactor.

3.2.7 Experimental design

Two identical bioreactor systems were set-up. After inoculation, both bioreactors were operated at 45^{o}C until granule settled bed height had grown to 2.0 cm. Thereafter, one bioreactor was kept at 45^{o}C and the other was increased to 70^{o}C. At 24 h intervals, the influent rate was increased as follows: day 1 : 75 ml/min, day 2: 90 ml/min, day 3: 105 ml/min, day 4: 120 ml/min, day 5: 135 ml/min, day 6: 150 ml/min; day 7: 165 ml/min, day 8: 180 ml/min; day 9: 195 ml/min, day 10: 210 ml/min, day 11: 225 ml/min. On each of the 11 experimental days, gas and sucrose measurements were determined for each of the following effluent recycles rates: 1.3 L/min, 1.6 L/min, 2.0 L/min, 2.3 L/min, 2.6 L/min, 2.9 L/min, 3.2 L/min, and 3.5 l/min. Effluent discharge rate or discharge force into the gas disengager was also a dependent of the effluent recycle rate.

3.3 Results and Discussion

3.3.1 Granule growth

Following the inoculation of the bioreactors, granule formation took place within 5 days after the Endo medium supply rate or influent rate had reached 4.5 L/h. To grow the granular bed, the influent rate was then increased every 24 h. At the end of 11 days, the settled bed had grown to 14 cm in the 45°C treatment and to 17 cm for the 70°C treatment. This corresponded to a total granule dry mass of 88 g for the 45°C treatment and 102 g granule dry mass for the 70°C treatment. At a degassed effluent recycling rate of 3.5 L/min, both granular beds at this stage could be expanded to fluidized volumes which occupied approximately 90% (4.5 L) of the bioreactor vessel volume, giving fluidized bed biomass densities of 19.5 and 22.7 g/L, for the 45°C and 70°C bioreactors, respectively.

3.3.2 Influence of temperature and effluent recycle rate on H_2 productivity

Figure 3.3 and 3.4 show the effects of temperature and effluent recycle rate on total H_2 output rates for the 45°C and 70°C bioreactors, respectively. At day 11, for the 45°C treatment as the effluent recycle rate was increased from 1.3 to 3.5 L/min, the total H_2 output for the bioreactor increased from 10.6 to 43.2 L/h, representing a 4.1-fold increase in H_2 productivity (Figure 3.3). Also, at day 11, for the 45°C treatment increasing the effluent recycle rates from 1.3 to 3.5 L/min resulted in an increase in the volumetric H_2 productivity from 2.1 to 8.7 L H_2/L/h or from 0.073 to 0.296 mol H2/L/h. In the 70°C treatment, at day 11, as the effluent recycle rate increased from 1.3 to 3.5 L/min, the total H_2 output for the bioreactor increased from 13.8 to 73.8 L/h, representing a 5.3-fold increase in H_2 productivity (Figure 3.4). In the 70°C treatment, at day 11, the volumetric H_2 productivities increased from 2.8 to 14.8 L H2/L/h or from 0.095 to 0.506 mol H_2/L/h as the effluent recycle rate was increased from 1.3 to 3.5 L/min. At the highest effluent recycle rate, an increase in the temperature from 45°C to 70°C resulted in a 171% increase in the volumetric H_2 productivity. At effluent recycle rates of 3.5 L/min, the specific H_2 productivities in terms of bacterial granule biomass were 0.491 and 0.724 L H_2/g h for the 45°C and 70°C bioreactors, respectively. On the 11th day, the influent rate or nutrient supply rate was 13.5 L/h (225 ml/min) which corresponds to a hydraulic retention time of 22.2 min for a working bioreactor bed volume of 5 L and 46.7 min for the total system volume (10.5 L). The hydraulic retention time for the combined highest influent rate (225 ml/min) and effluent recycle rate (3.5 L/min) was 1.34 min for the 5 L bioreactor working volume. The combined linear fluid flux through the fluidized granular bed was 1.24 cm/s (44.7 m/h). High linear fluid velocities through the fluidized granular bed played a significant role in increasing the volumetric H_2 productivity by facilitating the rapid removal of the dissolved hydrogen trapped in the liquid phase from the fluidized granular bed.

3.3.3 Influence of temperature and effluent recycle rate on % H_2 content

Over the 11 day period, the mean % H_2 content was 44.5 ± 2.58% for the 45°C bioreactor (Figure 3.5) and 67.3 ± 7.03% for the 70°C bioreactor (Figure 3.6). Increasing effluent recycle rates tended to increase the % H_2 content only in the 70°C bioreactor (Figure 3.6). With the 70°C bioreactor on days 10 and 11, the % H_2 content increased to 80% as the effluent recycle rates were increased to 3.5 L/min. Compared to the 70°C bioreactor, there

were no significant deviations from the mean value for % H_2 content in response to increasing effluent recycle rates in the 45°C bioreactor. On day 11, the average % H_2 content for the 45°C bioreactor over the range of effluent recycle rates was 47.3% compared to 70.6% for the 70°C bioreactor. An increase in the % H_2 content indicates an increase in the H_2 production efficiency or hydrogen yield (HY).

3.3.4 Influence of temperature and effluent recycle rate on hydrogen yield

Increasing % H_2 contents were associated with increasing HYs (Figure 3.5, 3.7, 3.6, and 3.8). HYs increased in response to increasing effluent recycle rates and also to increases in temperature. In the 45°C bioreactor, as the effluent recycle rates were increased from 1.3 to 3.5 L/min, HYs increased from 0.3 to 1.24 mol H2/mol glucose. In the 70°C bioreactor, as the effluent recycle rates were increased from 1.3 to 3.5 L/min, HYs increased from 0.41 to 2.2 mol H_2/mol glucose. Both the HY and HP values for the 70°C bioreactor were almost double the HY and HP values of the 45°C bioreactor. Increasing the temperature from 45 to 70°C would have promoted the forward reaction by increasing the standard Gibbs free energy ΔG°' for the reaction 3.1:

$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 4H_2 + 4H^+ + 2HCO^-_3$ Reaction 3.1

From -224.7 to -247.2 kJ/mol, making H_2 generation from glucose thermodynamically more favourable. It has also been reported that anaerobic H_2 consumption via an H_2 uptake hydrogenase was reduced with increasing temperature, resulting in a net increase in H_2 generation (Niu *et al*., 2011).

Currently, the low space/time yields (STYs) per unit volume has discouraged the commercialisation of biohydrogen production (Nath and Das, 2011). An increase in the STY for biohydrogen generation would require a simultaneous increase in both HP and HY. However, HYs for most high HP continuous dark anaerobic fluidized granular bed bioprocesses rarely exceeds 2.5 mol H2/mol glucose. Examples of experimental results with low HY and high HP results include: 1.3 mol H_2/mol glucose and 152.5 mmol H_2/L/h (O-Thong *et al*., 2008); 1.66 mol H2/mol glucose and 306.3 mmol H_2/L/h (Zhang *et al*., 2008a); 2.01 mol H_2/mol glucose and 380.8 mmol H_2/L/h (Lee *et al*., 2006). Experimental conditions that are necessary for the achievement of HYs equal to or greater than 3.0 mol H2/mol glucose, do not usually promote high HPs simultaneously. For example, dark anaerobic fermentations that produce HYs equal to 4 mol H_2/mol glucose, are strongly dependent on the

following experimental conditions: single species cultures, thermophilic temperatures, low substrate loading rates, low dilution rates, H_2 gas stripping by sparging with N_2, low hydrogen partial pressures and low bacterial biomass densities (de Vrije *et al*., 2007; Zeiden and van Niel, 2010). In general, high HPs have been achieved under the following experimental conditions: undefined mixed bacterial consortia, high substrate loading rates, high H_2 partial pressures, high dilution rates, and high bacterial biomass densities.

Increasing the 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. The anaerobic oxidation of the anions of alkanoic acids such as propionate and butyrate to acetate and H_2 becomes endergonic when the partial pressures of H_2 exceed 4 Pa or the dissolved H_2 concentration exceeds 0.024 lM (Lee and Zinder, 1988; Zinder and Koch, 1984). With a reduction in the H_2 partial pressure below 4 Pa, the oxidation of alkanoic acids and acetate becomes exergonic in the absence of H_2 consuming bacteria (Adams *et al*., 2006; Valentine *et al*., 2000), and HYs should then exceed the theoretical limit of 4.0 mol H2/mol glucose. A reduction in the H_2 partial pressures within the bioreactor to the levels necessary for achieving HYs equal to or greater than 4.0 mol H_2/mol glucose represents a major challenge in the design and operation of AFGBs.

- **Assessment of gas disengagement**

H_2 production efficiency is very sensitive to H_2 partial pressures. Efficient removal of dissolved H_2 from the fluidized granular bed through high rates of degassed effluent recycling increase the efficiency of H_2 production. In the methods section, it was proposed that the dissolved H_2 in the effluent stream should consist of two components: solubilised H_2 and non-solubilised H_2. Effective gas disengagement should remove most of the non-solubilised H_2 which consists chiefly of H_2 molecules trapped in a non-solubilised state in the liquid phase. Bubble production in the gas-disengager, as a consequence of high rates of effluent recycling through the gas-disengager, enhanced the transfer of H_2 from the liquid bulk phase to the gas phase by increasing the interfacial specific gas-exchange area between the two phases. However, complete removal of dissolved H_2 from the recycled effluent would be physically impossible without extreme sparging of N_2 gas through the gas-disengager. To increase HYs to 3.0 mol H_2/mol glucose in the AFGB system would require the reduction of the concentration of fully solubilised or dissolved H_2 in the recycled effluent,

leaving the gas-disengager to levels below the thermodynamic equilibrium concentration. This was not achieved in this experimental bioreactor system.

3.3.5 Sucrose consumption at high organic loading rates

On day 11 when the granular bed fluidization bed volumes occupying 90% of the bioreactor volume, the mean values for total sucrose loading rates of 223.4 g/h consumed at 45^{o}C and 70^{o}C bioreactors were 93% and 90% respectively. On day 11, the mean COD concentration of the influent was 19.5 g COD/L and the mean concentration of the total effluent COD for the 45^{o}C and 70^{o}C bioreactors were 4.8 and 4.6 g COD/L respectively (Table 3.1).

Table 3.1 COD mass balance on day 11 for an influent rate of 13.5 L/h and effluent recycle rate of 4.5 L/min. All values are the means of 3 replicates.

Bioreactor temperature	Influent COD g COD /L	Total effluent COD g COD/L	Total effluent soluble COD g COD/L	Effluent sucrose COD g COD/L	Effluent VFA COD g COD/L	Effluent Insoluble COD g COD/L
45 ^{o}C	19.5	4.76	3.92	1.16	2.76	0.84
70 ^{o}C	19.5	4.55	3.85	1.86	1.99	0.70

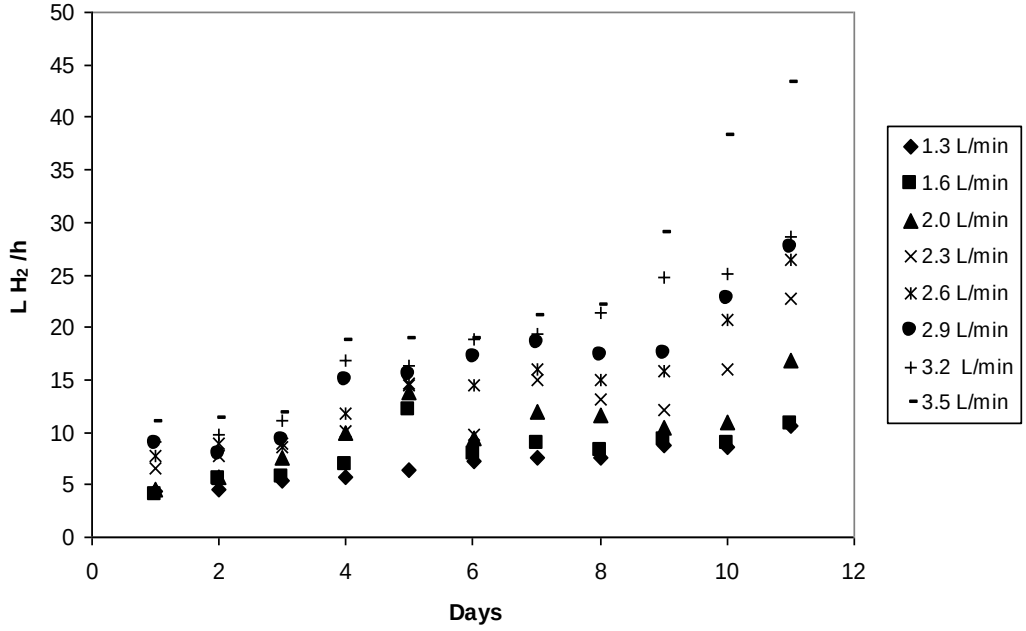

Figure 3.3 The influence of effluent recycle rates (L/min) on total hydrogen production at 45°C at increasing nutrient supply rates during granular bed growth.

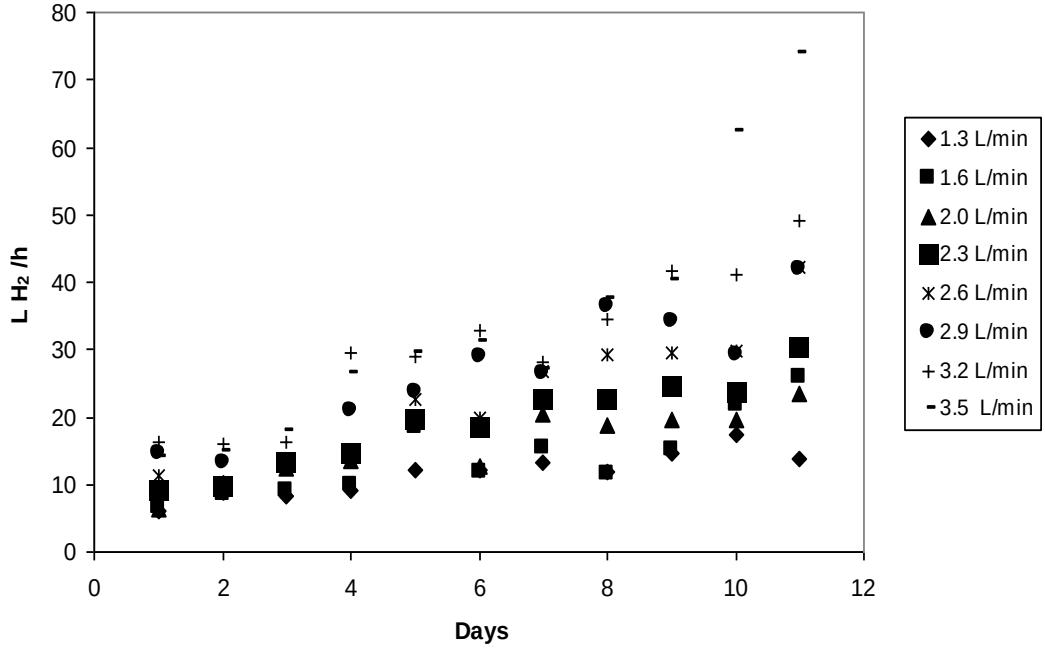

Figure 3.4 The influence of effluent recycle rates (L/min) on total hydrogen production at 70°C at increasing nutrient supply rates (L/h) during granular bed growth.

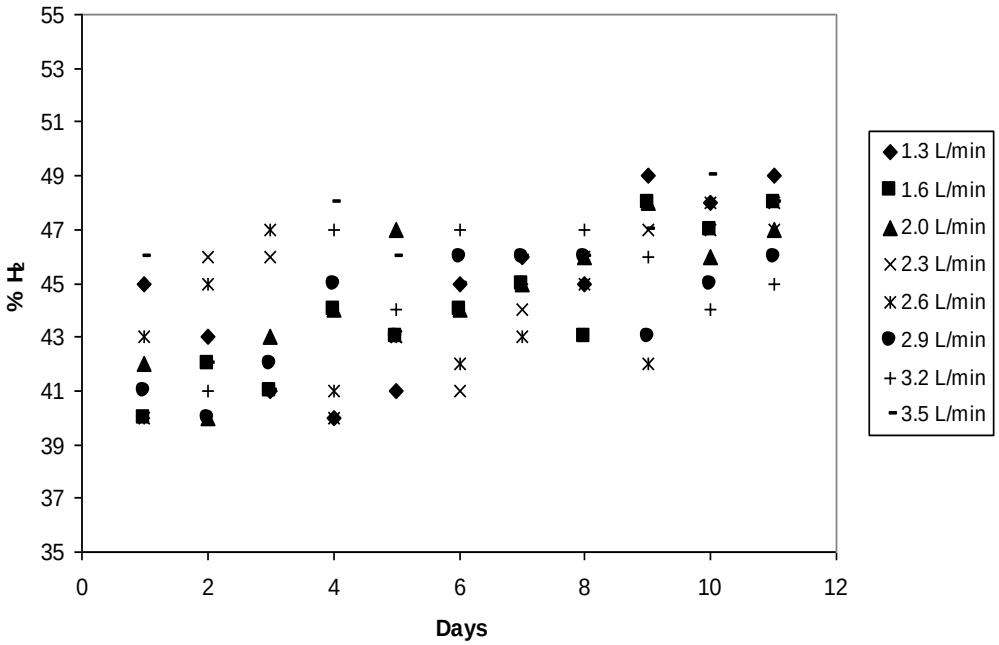

Figure 3.5 The influence of increasing effluent recycle rates on % H2 content at 45°C at increasing nutrient supply rates during granular bed growth.

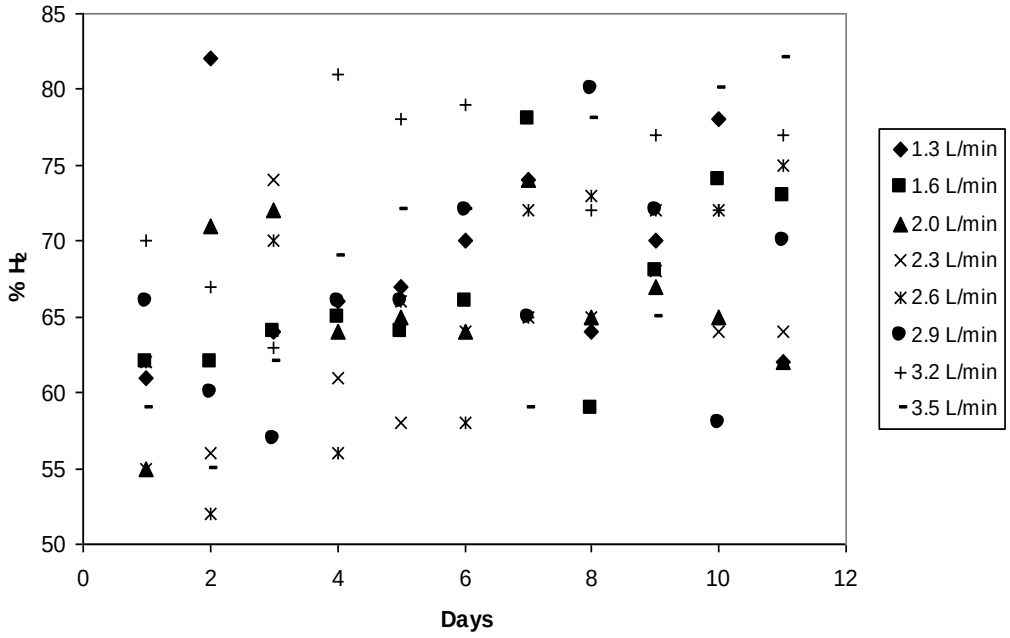

Figure 3.6 Influence of increasing effluent recycle rates on % H_2 content at 70°C at increasing nutrient supply rates during granular bed growth.

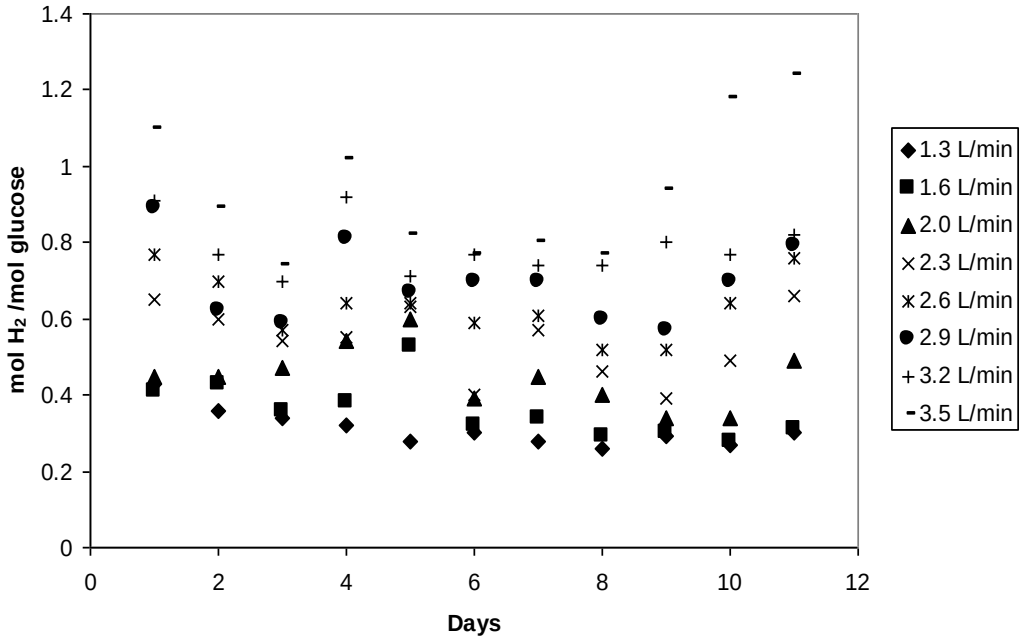

Figure 3.7 The influence of increasing effluent recycle rate on hydrogen yield at increasing nutrient supply rates during granular bed growth at 45°C.

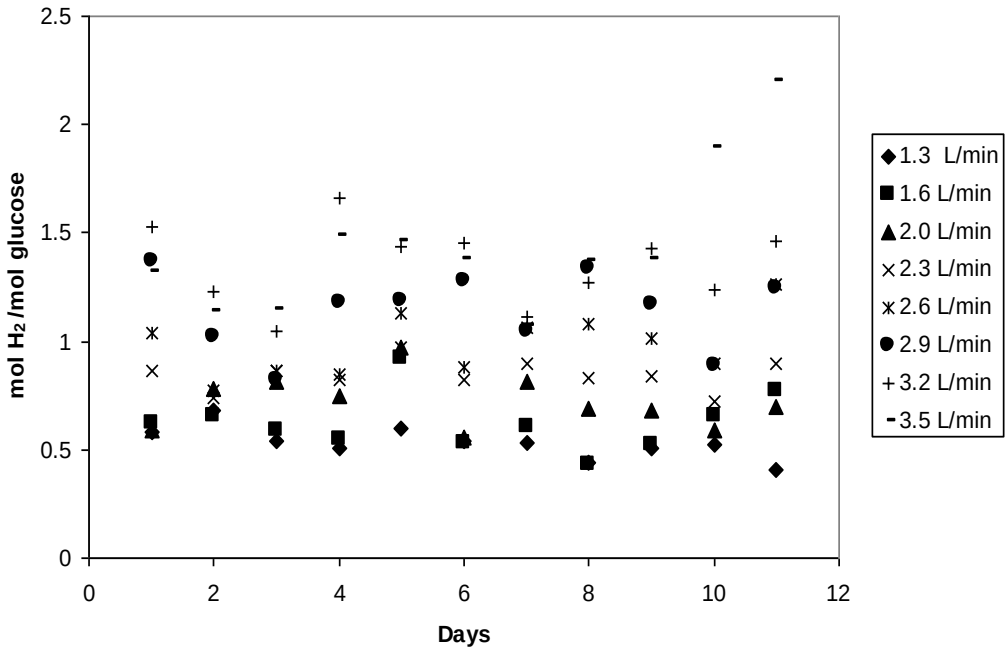

Figure 3.8 The influence of increasing effluent recycle rates on hydrogen yield at increasing nutrient supply rates during granular bed growth at 70°C.

3.4 Conclusion

Effluent discharged at high flow rates from the bioreactor generated high levels of bubble formation through effluent turbulence and liquid cavitations within the gas-disengager, resulting in the release or disengagement of non-solubilised H_2 from the effluent. Any increase in the quantity of non-solubilised H_2 removed from the effluent recycled through the bioreactor system, would shift the reaction equilibrium in favour of H_2 production. This hypothesis explains why high rates of effluent recycling through the gas disengager increased both volumetric hydrogen productivities and hydrogen yields.

Chapter 4
Influence of increasing temperatures and the bioreactor volume to effluent recycle rate ratio on hydrogen productivities and hydrogen yield

4.1 Introduction

Is it thermodynamically possible to achieve high volumetric hydrogen productivities (2 900 L H_2 /m^3/h) and maximum hydrogen yields (> 4 H_2 /mol glucose) simultaneously? In order to define benchmarks, the minimum economically viable bioreactor volumetric HP could be made equal to the minimum acceptable work performed by the bioreactor on some load. A bioreactor's volumetric power generating capacity, or work rate on a load, could be expressed in terms of the H_2 supply rate required for the operation of a 5 kW fuel cell. A 5 kW hydrogen fuel cell which has the capacity to power a house requires a fuel supply rate not less than 2 900 L/h. Expressed on a volumetric basis this H_2 rate is equivalent to 120 mol H_2/m^3/h. This value could be used as the minimum HP benchmark or threshold for evaluating the economic viability of a given biohydrogen generation process. If capital cost reduction is the economic objective in biohydrogen commercialisation then the bioprocess with the highest possible HP should be selected. This suggests that for a bioprocess to be commercially viable the bioreactor unit volume required to perform useful work on a 5 kW fuel cell should not be greater 1 m^3. Therefore the HP for a biohydrogen bioprocess should not be less than 120 mol H_2/m^3/h. The next problem concerns the HY. To increase the commercially viability of biohydrogen generation the efficiency of the bioprocess also needs to be maximised.

To date the thermodynamic or substrate conversion efficiencies for dark anaerobic biohydrogen production in all recent high rate continuous bioreactor processes have not exceeded 3 mol H_2 /mol glucose, which represents 75% of the so-called theoretical maximum efficiency of H_2 production (Ngoma *et al*., 2011). The reason for this is that the experimental conditions under which HPs have reached levels between 380 mmol H_2 /L/h (Lee *et al*., 2006) and 506 mmol H_2 /L/h (Ngoma *et al*., 2011) does not also favour the simultaneous achievement of HYs equal to or greater than 3.0 mol H_2/mol glucose.

Achievement of HYs equal or greater than 3.0 mol H_2/mol glucose in bioreactor experiments have always been dependent on the following operational conditions: monocultures, thermophilic temperatures, low substrate loading rates, low dilution rates, H_2 gas stripping by sparging with N_2, maintenance of low H_2 partial pressures (< 100 Pa) and low bacterial biomass densities (de Vrije, *et al*., 2007; Zeiden and van Niel, 2010). Achievement of high HPs depends on the following operational conditions: undefined multispecies bacterial consortia, high substrate loading rates, high H_2 partial pressures, high dilution rates, and high bacterial biomass densities (Barros *et al*., 2010; Lee, *et al*., 2006; Ngoma, *et al*., 2011; Zhang, *et al*., 2008b). High volumetric hydrogen productivities are usually correlated with high H_2 partial pressures within the bioreactor system (Ngoma *et al*., 2011). High H_2 partial pressures (>100 Pa) within the bioreactor system are usually correlated with low HYs, that is, HYs below 3 mol H_2/mol glucose. Low HYs and high H_2 partial pressures are associated with the accumulation of the VFAs (acetate, propionate and butyrate). Under high H_2 partial pressures the aggregated reaction for the anaerobic oxidation of glucose by a multispecies bacterial consortium can be expressed as follows (Reaction 4.1):

$$C_6H_{12}O_6 + tH_2O \rightarrow uCH_3COO^- + vCH_3CH_2COO^- + wCH_3CH_2CH_2COO^- + xH^+ + yHCO_3^- + zH_2$$ Reaction 4.1

with the different VFAs being produced in proportions corresponding to u:v:w, where u, v, and w are never zero. When butyrate and propionate have been generated the HYs are never greater than 2.0 H_2 /mol glucose (Table 4.1), even when HPs have been greater than 2 900 L H_2 /(h.m^3).

In this chapter the influence of temperature, bioreactor volume and effluent recycle rate on HP and HY was investigated. In chapter 3 the bioreactor working volume was 5.0 L (V) and the maximum effluent recycle rate (R) was 3.5 L/min. Under these operational conditions, where V/R was 1.43 min the HY was always below 3.0 mol H_2/mol glucose. Also, in this chapter it was observed that by decreasing V/R to 0.94 min both HP and HY were both simultaneously increased.

4.2 Materials and Methods

The Endo formulation (Endo *et al*., 1982; Ngoma *et al*., 2011) was used as the nutrient medium for inoculum preparation and for the bioreactor experiments. The medium contained 17.8 g sucrose/L and the following mineral salts (g. L^{-1}): NH_4HCO_3 6.72, $CaCl_2$ 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}g.

4.2.1 Inoculum preparation

Fresh sewage sludge was obtained from the overflow outlet of the mesophilic anaerobic digestions at the Olifantsvlei wastewater treatment works (Johannesburg). Sewage sludge samples were incubated at 90ºC for 2 hours. After the heat treatment, the pH of the sludge was reduced to pH 2.0 with 0.1 N HCl. Samples were maintained at this pH in sealed airtight bottles for 12 h at room and then readjusted to pH 7.0 by adding Endo medium (50% v/v). After this treatment, the inoculum was applied to the bioreactor.

4.2.2 Bioreactor design and set-up

The bioreactor system comprised of two main components a tubular bioreactor vessel for the bacterial granular bed and a tubular gas-disengager (Figure 4.1). Clear Perspex hollow tube was used for the construction of the tubular bioreactor (internal diameter (ID): 80 mm; height (H): 600 mm). The working volume for the bioreactor's fluidized bacterial granular bed was 3 L and the volumetric hydrogen productivity was expressed in terms of this volume rather than the total working volume of the entire bioreactor system. At the base of the bioreactor, the clear Perspex cylinder was connected to a conical-shaped diffuser (ID: 80 mm and H: 150 mm) made from PVC which functioned as 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 diffuse was filled with a 100 mm layer of 5 mm glass beads. Positioned in the middle of the diffuser were 4 inlet ports (ID 5 mm) with each inlet arranged at 90^o with respect to the two other inlets on each side. Nutrient medium (influent stream) was supplied directly into the upper glass bead layer via 4 inlet ports. The effluent overflow from solid-liquid separator was decanted into a gas-disengager which consisted of a gas collection cylinder (H: 200 mm and ID: 150 mm) connected to 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 at 45 rpm which gave a volumetric of 3.2 L/min. The second effluent outlet drained the effluent overflow from the gas-disengager. The lid of the gas collection cycle contained a gas outlet port connected to a gas meter (Ritter drum-type gas meter TG 05/3). The liquid-gas separator or gas-disengager had a working volume of 1.54 L and the total fluid occupied volume of interconnecting piping stood at 0.934 L. Total fluid containing working volume of bioreactor system (bioreactor bed, solid-liquid separator, gas-disengager, diffuser, and piping) represented 5.5 L. A Watson-Mallow Bredel (model 520U) peristaltic pump (Falmouth, UK) was used to pump the Endo nutrient into the bioreactor. Bioreactor and gas-disengager temperatures were maintained at the various operational temperatures (45 ºC, 55 ºC, 60 ºC, 65 ºC and 70 ºC) by re-circulating water from a heated water bath through the bioreactor and gas-disengager water jackets. Watson-Mallow Bredel (model 520U) peristaltic pumps were used to pump the Endo nutrient into the bioreactor.

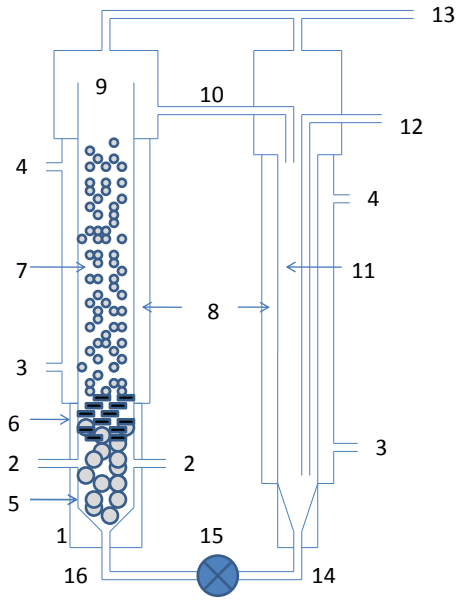

Figure 4.1 Bioreactor prototype for the simultaneous achievement of high HPs and HYs. Diagram labels: 1 – inlets manifold; 2 – influent inlets; 3 – water jacket inlets for heat exchanger; 4 – water jacket outlets for heat exchanger; 5 – bed of glass bed (5 mm) in effluent/influent diffusion and cavitation 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; effluent recycle outlet pipe; 14 effluent gas disengagement, 15 – effluent recycle pump; 16 – effluent recycle inlets.

4.2.3 Effluent gas disengagement

As reported in a previous study the effluent gas disengager is used to reduce to the concentration of H_2 trapped in the effluent to its thermodynamic equilibrium concentration (Ngoma *et al*., 2011). This was accomplished by facilitating the maximum transfer or release of H_2 from the liquid phase within the gas disengager to the vapour phase, which turn was being continuously exhausted from the gas disengager. High rates of effluent recycling between the bioreactor and the gas disengager generated a high degree of fluid turbulence and cavitations within the gas disengage tube. This process facilitated the release of super saturated dissolved H_2 through bubble production. Efficient removal of H_2 trapped in the effluent phase by the gas disengagement would increase the efficiency of the bioreactor.

4.2.4 Bacterial granule induction

On top of the glass bead, bed a 100 mm bed of CAC particles (diameter = 2.5 mm and length = 5.0 mm) was used to facilitate the induction of bacterial granulation in the bioreactor (Lee *et al*., 2004; Ngoma *et al*., 2011). Prior to its use, the CAC was first washed with distilled water to remove all the suspended fine particles and then sterilised by autoclaving for 20 minutes. Sufficient CAC was added to the bioreactor to give a settled bed height of 100 mm. Endo medium (3.0 L) and treated seed inoculum (2.0 L) were added to the bioreactor system. Following inoculation, the bioreactor was operated on a batch effluent-recycle mode for 48 h at 45^{o}C to acclimatise the bacteria and allow for their attachment to the CAC. After this acclimatisation period, the bioreactor operation was switched to continuous effluent recycle mode with an initial HRT of 8 h. The HRT was then gradually decreased over 2 day intervals by increasing the nutrient medium supply rate. As the HRT was decreased from 8 to 4 h, the growth and development of bacterial biofilm on the CAC particles became visible. With further decreases in the HRT below 4h, the biofilm growth increased and bacterial granules began to form and accumulate at the surface of the expanded CAC bed. Once granule formation had been initiated, further reductions in the HRT to between 2 and 1.6 h resulted in the rapid growth and expansion of the granular bed. Granule induction, growth and development were carried out at 45^{o}C. The HRT was then gradually decreased over 2 day intervals by increasing the nutrient medium supply to a final rate of 4.5 L/h. After granules had grown to a settled bed height of 140 mm the temperature was increased from 45^{o}C in the following order: 50ºC, 55ºC, 60ºC, 65ºC and 70ºC.

4.2.5 Analytical techniques

H_2 analysis: Gas chromatography was used to analyse % 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 40µl. The following formula was used for converting total bioreactor gas flux (L/h) to mmol H_2/h,

$$\frac{\Delta H_2}{\Delta t} = \frac{P\left[\left(\%H^{GC}\right)\frac{\Delta V}{\Delta t}\right]}{RT}. \qquad \text{Equation 4.1}$$

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.

4.2.5.1 VFA and ethanol analysis

The concentrations of acetate, propionate and butyrate in the effluent were determined by gas chromatography using the Varian 3300 FID GC as equipped with a CP Wax 58 (FFAP) capillary column (25m x 0.53 mm). Initial column temperature was set at 50°C for a holding time of 2 min. The oven temperature was increased to 190°C at 15 °C/min and then held at 190°C for 5 min. The temperatures of the injector and the detector were 250°C and 260°C, respectively. Helium was used as the carrier gas. Preparation of samples involved centrifugation at 12 000 rpm for 5 min followed by acidification with orthophosphoric acid (30 µl 17% phosphoric acid per 1.0 ml of sample). The samples were filtrated through 0.2 µm membrane before injecting a 2.0 µ sample. Lactose and ethanol were analysed by HPLC using a Waters Shodex ion pak KC811 column at 80^{o}C with 3 mM H_2SO_4 as the mobile phase (1ml/min).

4.2.5.2 Sucrose analysis

The concentration of sucrose in the reactor effluent was determined according to the standard sucrose-resorcinol method (Kerr *et al*., 1984). The concentration of sucrose in the reactor effluent and feed was measured calorimetrically 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. 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 at 80^{o}C for 8 min. Two miles of H_2O was added later 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 the method described above.

4.2.5.3 Experimental design

To make the temperature treatments comparable, the settled bed height was maintained at 14 cm by adjusting the nutrient supply rate. During experimental measurements, the nutrient supply rate was maintained at 4.5 L/h and the effluent recycle rate was maintained at 3.2 L/min. The starting temperature was 45^{o}C for granule induction and once the granular bed was established the temperature was increased. At 24 h intervals, the temperature was increased from 45^{o}C in the following order 50 ºC, 55 ºC, 60 ºC, 65 ºC and 70 ºC. All gas and sucrose measurements were replicated three times. It was found that 24 h was sufficient for the bacterial granules to acclimatise to each temperature increase.

4.3 Results and Discussion

4.3.1 Temperature acclimatisation

Application of nutrient influent rates of 4.5 L/h and degassed effluent recycle rates of 3.2 L/min maintained the bacterial granular bed in a state of fluidization with the fluidized bed occupying more than 75 % of the bioreactor volume. No methane was detected at any of the temperatures. Granule growth and hydrogen production were maintained at all temperatures. Biomass density was maintained at 41 g /L which corresponded to a settled bed height of 14 cm. When fluidized to 75 % of the bioreactor's working volume, the fluidized biomass density was equal to 18.2 g BM /L (where BM is bacterial biomass).

4.3.2 Total bioreactor H_2 generation

Total bioreactor HP for the 3.0 L bioreactor working volume increase by two fold from 10.56 L H_2 /h (0.362 mol H_2/h) to 21.58 L H_2 /h (0.74 mol H_2/h) as the temperature was increased from a mesophilic temperature of 45^{o}C to the extreme thermophilic temperature of 70^{o}C. The increase in H_2 production was linear in response to increasing temperature with a rate of increase equal to 0.4935 L H_2/h/^{o}C ($y = 0.4935x – 12/259$, $R^2 = 0.9582$) (Figure 4.2).

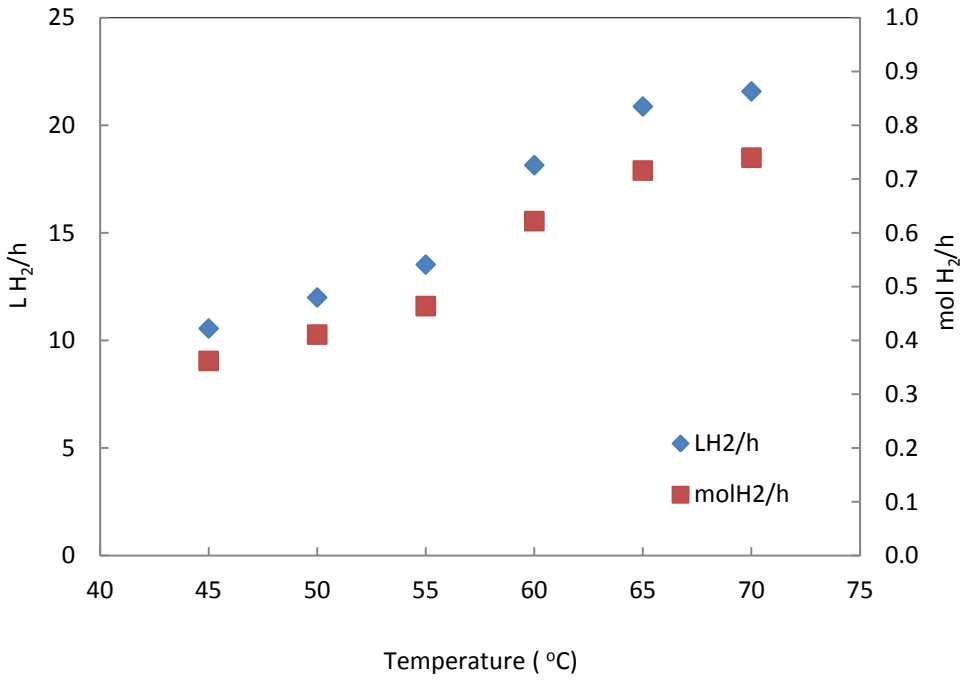

Figure 4.2. The influence of temperature on total HP

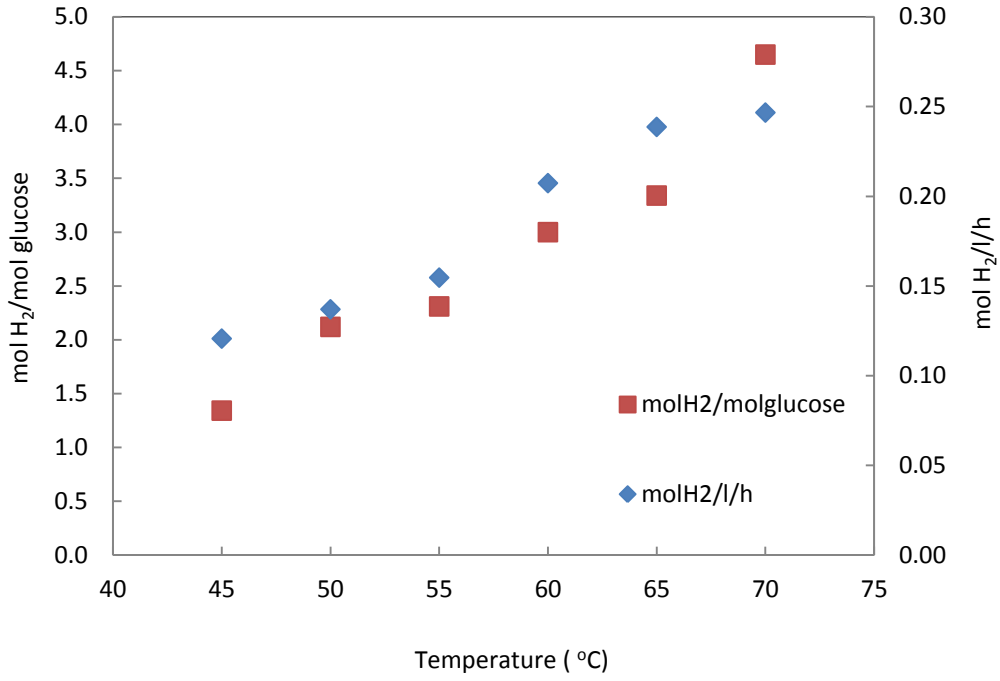

Figure 4.3 The influence of temperature on total HP and HY

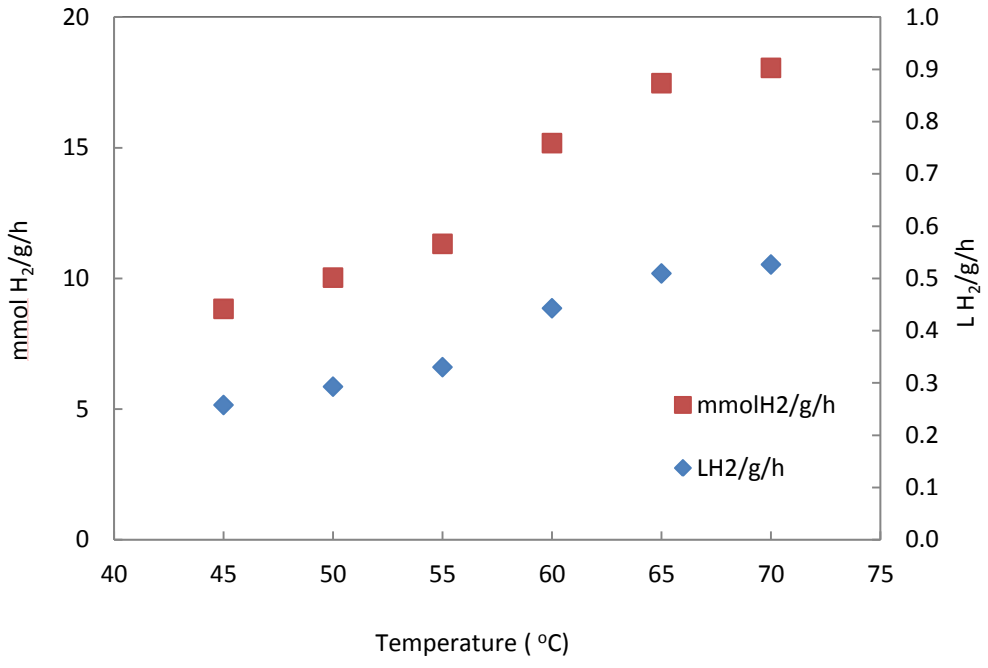

Figure 4.4 The influence of temperature on SHP (specific H_2 production rate per gram BM).

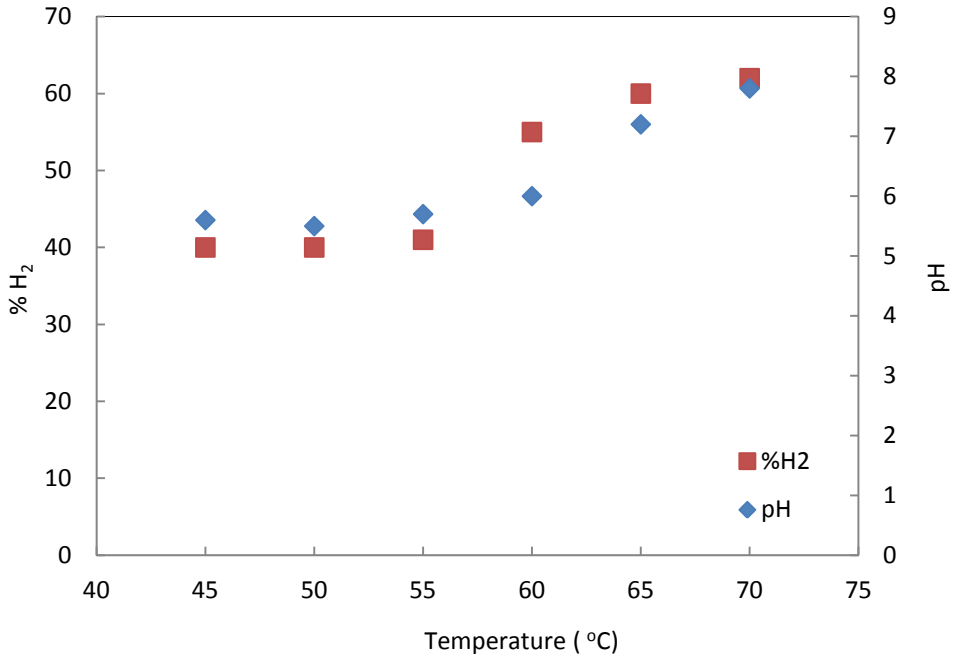

Figure 4.5. The influence of temperature on % H_2 and pH

4.3.3 Volumetric hydrogen productivity

Volumetric HP also doubled from 3.42 L H_2 /L/h (0.121 mol H_2 /L/h) to 7.19 L H_2 /L/h (0.247 mol H_2 /L/h) as the temperature was increased from 45°C to 70°C (Figure 4.3.). The HP increased linearly with temperature. Recently reported thermophilic HPs have ranged from 3.72 L H_2/L/h, calculated from the 0. 152 mol H_2/L/h value reported by O-Thong, *et al*., (2008), to 14.8 L H_2/L/h (0.506 mol H_2/L/h) reported by Ngoma, *et al*., (2011).

4.3.4 Hydrogen yield

A 3.47 fold increase in HY occurred with an increase in temperature from 45°C to 70°C (Figure 4.3). Expressed in terms of glucose, this represents an increase from 1.34 mol H_2 /mol glucose to 4.65 mol H_2 /mol glucose. The current HY at 70°C was 3.75 fold higher than the previously reported value of 1.24 mol H_2 /mol glucose. A factor which contributed to this increase in HY was the substantial decrease in the bioreactor working volume (V, L) to degassed effluent recycle ratio (R, L/min). In the previous study, V/L = 1.43 min. In this study, V/L = 0.94 min, which represents a 65.7% reduction in the bioreactor's fluid volume phase turnover time.

4.3.5 Specific hydrogen productivity

Specific HY for the fluidized granular bed increased from 0.258 L H_2 /g/h (8.83 mmol H_2 /g/h) at 45^{o}C to 0.526 L H_2 /g/h (18.03 mmol H_2 /g/h) at 70^{o}C (Figure 4.4).

4.3.6 Percentage hydrogen content

Percentage H_2 content increased from 40% at 45^{o}C to 62% at 70^{o}C (Figure 4.5). As the temperature was increased to 60^{o}C the H_2 increased to 55%. At 65^{o}C and 70^{o}C H_2 content increased to between 60% and 62%.

4.3.7 VFAs and ethanol

With increasing temperature, concentrations of the VFA (acetate, butyrate and propionate) decreased (Figure 4.6). Similarly with increasing temperatures the concentrations of lactate, succinate and ethanol declined sharply (Figure 4.7). At temperatures greater than 60^{o}C succinate and ethanol concentration fell to zero. The concentrations of the VFAs fell exponentially with increasing temperatures. The rise in the HY and pH (Figures 4.3 and 4.5) was consistent with exponential decline in the concentration of the VFAs. In reaction 4.1 for the anaerobic oxidation of glucose, a decline in the values of u, v, and w for VFAs is consistent with a rise in the value of z for H_2. Patterns of VFA production shown in Figure 4.6 differ from other trends occurring in other anaerobic fluidized bed bioreactors as summarised in Table 4.1. At low HRT (between 0.5 and 1 h) and with substrate concentrations equal to or greater than 10 g/L, the average concentration of acetate, propionate and butyrate were 42.6 mM, 4.55 mM and 29.2 mM, respectively (Table 4.1). High butyrate and propionate concentrations were generally associated with low HYs (Table 4.1).

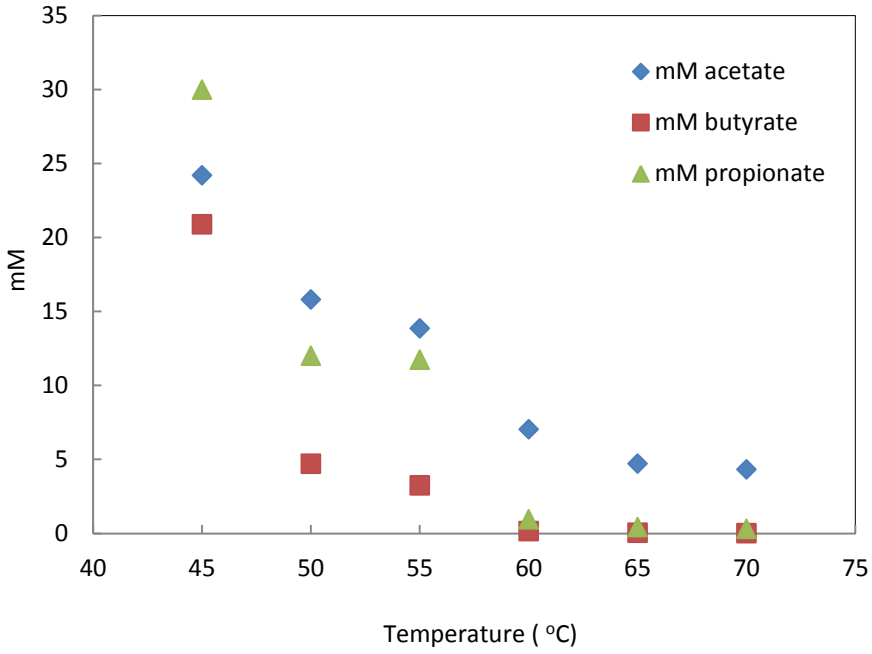

Figure 4.6 The influence of temperature on acetate, propionate and butyrate production.

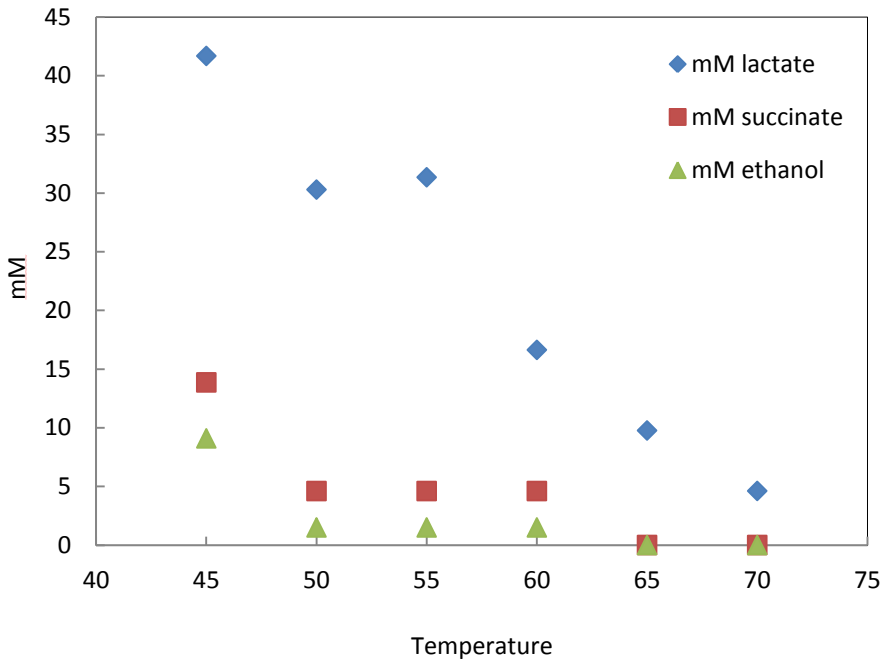

Figure 4.7 The influence of temperature on lactate, succinate and ethanol production.

4.3.8 Lactate, Succinate and Ethanol

At lower temperatures it appears that the lactate formation pathway was favoured (Figure 4.7). At lower temperature H_2 generation appeared to be sensitive to H_2 partial pressures, and this would favour the flow of electrons into the lactate producing pathway. However as temperatures were increased to the extreme thermophilic levels lactate production fell. In addition, succinate levels fell. Succinate is a key intermediate of the propionate synthesis pathway, and thus its decline as temperatures increased could be taken as an indication of propionate oxidation (Figure 4.7)

Table 4.1. The influence of pH, temperature, HRT and substrate loading rate on $\%H_2$, HP, HY, VFAs, lactate and ethanol in various fluidized granular bed bioreactors. s – sucrose, g – glucose.

T	Substrate	HRT	pH	$\%H_2$	HP	HP	HY	Acetate	Propionate	Butyrate	Lactate	Ethanol	Reference
^oC	g/l	h			L H_2/L/h	mmolH_2/ L/h	mol H_2/mol gluc	mM	mM	mM	mM	mM	
60	20.0(s)	0.75	5.5	55 - 60		152.5	1.3	42.2	2.4	44.8	0.5	9.2	O-Thong *et al*., 2008
35	17.8 (s)	0.5	6.7	41.7	9.31	380	1.96	46.9	11.4	34.2	-	-	Lee *et al*., 2006
37	10 (g)	1	4.0	59.2	1.25		1.19	20.2	1.2	15.5	-	6.8	Zhang *et al*., 2007a
37	20 (g)	1	4.0	59.2	2.30		1.18	39.1	1.9	25.4	-	11.8	
37	30 (g)	1	4.0	59.2	2.36		1.10	42.2	2.5	26.0	-	13.6	

4.4 Conclusion

In Chapter 3, HY increased with increasing effluent recycling rates. In that study, it was proposed that high rates of effluent recycling achieved two essential process goals which were necessary for increasing hydrogen yield. The first process goal was the rapid physical removal of H_2 trapped within fluid phase surrounding the bioreactor fluidized granular bed. The second process goal was the efficient effluent gas disengagement brought about by discharging the effluent at a high flow velocity from the bioreactor into the gas disengager tube. For effluent gas disengagement to be efficient, it would have been necessary for the process to remove most of the supersaturated concentration of dissolved hydrogen, H_2^L (mol), from the effluent before it was recycled back into the bioreactor. The H_2 content of the effluent recycled back into the bioreactor would correspond to the thermodynamic equilibrium dissolved hydrogen concentration, H_2^{L*} (mol), which would have been impossible to remove completely from the effluent stream in the gas disengager. So it was assumed that the effluent recycled back into the bioreactor would contain the thermodynamic equilibrium concentration of dissolved hydrogen. The linear velocity of the recycled effluent through the quasi-stationary fluidized granular bed was 1.06 cm/s which is considerably higher than the H_2 volumetric mass transfer coefficient (k_L).

In about 56 seconds, the entire fluid volume supporting the fluidized granular bed was displaced from the bioreactor with fresh degassed effluent mixed with influent nutrients, while the granular bed remained behind in the bioreactor. Recycling of degassed effluent at this velocity through the fluidized granules brings about the continuous and rapid displacement of H_2 containing bubbles and dissolved H_2 from the bioreactor bed. A degassed liquid volume flux of 3.2 L/min passing through the 2.309 L working volume corresponding to the fluidized granular bed would be 16 to 8.9 fold greater than the 0.2 L/min (50^oC) or the 0.36 L/min (70^oC) H_2 gas volume flux generated by the granular bed. Thus the physical rate of the removal of H_2 trapped in the liquid phase was considerably greater than the rate at which H_2 was being generated by the granules embedded in the liquid phase. Also an increase in temperature inhibits the H_2 consuming hydrogenases, thereby increasing the net flux of H_2 from the granules into the mobile fluid phase (Ngoma *et al*., 2011). In addition, according to Le Chatelier's principle, under these operating conditions, the high rates of H_2 removal by the mobile fluid phase would make the ΔG'more negative not only for the

anaerobic oxidation of glucose, but also for the anaerobic oxidation of acetate, propionate and butyrate, in the absence of H_2 consuming bacteria. This would also add to the net flux of H_2 into the mobile liquid phase from the quasi-static fluidized granular bed. Thus the combination of high temperatures and a low V/R quotient appears to be the necessary bioreactor operation conditions for the simultaneous achievement of high HPs and HYs.

Chapter 5
Denaturing gradient gel electrophoresis (DGGE) investigation of bacterial community composition dynamic

5.1 Introduction

Prokaryotes which can be found in all habitats of the Earth, play an important role in the biogeochemical cycles of the biosphere and are involved in the recycling of chemical elements such as carbon and nitrogen (Robe *et al*., 2003). Most prokaryotes are capable of forming stable aggregate communities known as biofilms. In healthcare environments, related species of *Staphylococcus aureus* display similar phenotypic characteristics making the identification and differentiation by traditional culture techniques difficult. In addition, such species are recalcitrant to various antibiotics treatments (Da silva *et al*., 2003); however, some species could provide valuable molecules for industries (Robe *et al*., 2003). For example, only 1% of microbes found in environmental samples are culturable (Schneegurt *et al*., 2003). Therefore, the switch to appropriate and relevant sequencing techniques for the identification of aero-anaerobic bacteria, mycobacteria and fungal species is required. Sequence analysis of the ubiquitous ribosomal RNA (rRNA) has become the ideal tool for classification, detection, and evaluation of the microbial evolutionary relatedness (Hung *et al*., 2007). The advantage of the 16S rRNA gene sequence allows for a better identification of poorly described and rarely isolated strains (Clarridge, 2004). This molecular technique is routinely used in the food industry (Handschur *et al*., 2005), clinical studies (Knapp, 2005) and for microbial ecology studies (Muyzer and Smalla, 1998) for the identification of novel pathogens and uncultured microbes (Ercolini, 2004).

A popular molecular method for mapping environmental samples is the DGGE which analyzes polymerase chain reaction (PCR) products of the 16S ribosomal RNA (rRNA) fragments. These fragments are amplified using a specialised forward primer that contains a GC-rich region attached to the 5' end. The increasing gradient of denaturants allows for the separation of fragments based on their sequence differences. Theoretically, each band in one lane should represent a single microbial strain (Gafan and Spratt, 2005). Although this technique is highly advantageous (quicker and less laborious approach) for descriptive and

comparative analysis, it has a few flaws. In fact, DGGE has three main shortcomings: Firstly, single bands however do not represent single strains due to heteroduplexes, two sequences that differ in a few nucleotides can anneal. This often occurs during PCR amplification (referred to as PCR artifacts) (Sekiguchi *et al*., 2001). These PCR artifacts can lead to a misinterpretation of results and inaccurate conclusions. Secondly, gel-recovered DNA cannot be sequenced due to overlapping sequences or homoduplexe sequences that share the same mobility and co-migration due to same melting behaviour (Muyzer and, Smalla, 1998). Lastly, when two bands on a DGGE gel are physically close to one another, it is difficult to excise one band without encroaching the second one, thus contaminating the DNA sequences of the two. This technique can provide information on the predominant species in a community and analyze multiple samples simultaneously (Miller *et al*., 1999; Ding *et al*., 2011).

The aim of this chapter is therefore, to identify the biohydrogen producing microbial community. An AFGB containing CAC induced granules (Lee *et al*., 2003, 2004) was designed as described in chapter 3 and operated under 40°C and 70°C. Bacterial populations in the fermentative H_2 production bioreactor was analyzed using PCR-DGGE and scanning electron microscopy. The H_2 production results were compared with the information regarding microbial community structure to obtain a better understanding of the fermentative H_2 production.

5.2 Materials and Methods

5.2.1 Molecular approach

Molecular techniques have provided powerful tools to investigate microbial community in the bioreactor by overcoming the problems associated with the traditional cultivation dependent methods (Amann *et al*., 1995; Angenent *et al*., 2002; Calli *et al*., 2005; Ding *et al*., 2011; Lee *et al*., 2007; Ueno *et al*., 2001b). These techniques constitute: DNA Extraction, PCR amplification, Agarose gel electrophoresis, DGGE analysis and Phylogenetic analysis.

5.2.1.1 DNA Extraction

The samples were subjected to eubacterial 16S rDNA-targeted genes extraction followed by their sequencing. Duplicate samples of bioreactor carrier (0.2–0.5 g each) and liquid (1 ml each from bioreactor recycle line) were taken during the operation and stored at -20 °C. Bioreactor liquid mixed with granules was carefully removed from carrier samples by

pipetting before storing. Prior to DNA extraction of bioreactor, 1 ml of sample was pipetted into 12 eppendorfs tubes and micro-centrifuged at 10 000 rpm for 5 minutes and the supernatant was disposed. Pellets were pooled together and DNA was extracted following the method described by the ZymoResearch Fungal/Bacterial DNA kit according to the manufacturer' s instructions (Inqaba Biotechnical Industries, South Africa). 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 for 5 minutes, followed by a centrifugation at 10 000 x g for 1 minute. Four hundred microliters of the upper aqueous phase was transferred into a Zymo-Spin IVTM Spin Filter in a Collection Tube and centrifuged at 7 000 x g for 1 minute, 1200 µl of Fungal/Bacterial DNA Binding Buffer was then added to the subsequent filtrate where 800 µl of the mixture was transferred to the Zymo-Spin IITM Column in a new collection tube; followed by centrifugation at 10 000 x g for 1 minute, and a pre-wash DNA was performed 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 IITM 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. The purity of DNA was assessed on a nanodrop which measured the A260\A280 for protein impurities and other contaminants.

5.2.1.2 PCR amplification

Partial bacterial 16S rDNA gene amplifications were carried out in a reaction volume of 50 µl, containing: 25 µl PCR Master Mix (# K 0171, Fermentas), 1µl template DNA, 22 µl nuclease free water (# K 0171, Fermentas) and 1 µl of each oligonucleotide primer. The fermentas forward "sense" EUB968F (5'-AAC GCG AAG AAC CTT AC- 3') and the fermentas reverse "antisense" UNIV1392R (5'-ACG GGC GGT GTG TRC- 3'). The amplification profile was carried out using a Gene Amp PCR system 2700® thermocycler, using the following cycling conditions: One cycle of 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 1 minute 30 seconds, with a final cycle of extension at 72°C for 7 minutes.

5.2.1.3 Agarose gel electrophoresis

After DNA amplification, 5µl PCR products, 1.5 µl of tracking dye (# R0629, Fermentas) and 5 µl DNA ladder (# SM098, Fermentas) were loaded on a 1 % agarose gel for DNA analysis, separated by electrophoresis at 80 Volts for 4 hours, stained with ethidium bromide, visualised with ultraviolet light and photographed with Polaroid camera. The presence of a single bright band for each sample indicated successful amplification. The size of the fragment was determined using a 1Kb ladder on a 1% agarose gel.

5.2.1.4 DGGE analysis

The DGGE was performed with a BioRad DCode^{TM} Universal Mutation Detection System (BioRad Laboratories USA). Separation of the amplified sequences was obtained using 6 % (m/v) polyacrylamide gels with a denaturing gradient of 40–60 % (100 % denaturants gels defined as 7 M urea and 40 % deionised formamide) (Bio-Rad Laboratories USA). In each well, 200 ng PCR products were loaded and electrophoresis was performed at a constant voltage of 160 V for 5 hours in 7L 1× TAE running buffer at 60°C in the DGGE tank (Bio-Rad, USA). Gels were stained in 250 ml 1× TAE buffer with 20 µl 10 mg/ml ethidium bromide for 10 minutes and then distained in 250 ml 1× TAE buffer for 15 minutes this time without ethidium bromide. The gels were photographed under ultraviolet light (UV) using a Bio-Rad gel Doc XR system (Muyzer *et al*. 1993). 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 of the supernatant was used as template DNA in the re-amplication by PCR using universal 1392R and EUB968F primer, however, without a GC-clump. The resulting PCR product was sent for sequencing to Inqaba, South Africa. Primers EUB 968 forward with GC clamp: CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGG and UNIV1392 reverse primer were used to amplify the 16S rDNA from the purified plasmid DNA. PCR reactions and conditions were as described in section 5.2.1.2.

5.2.1.5 Phylogenetic analysis

Sequences were submitted to the National Centre for Biotechnology Information (NCBI) to determine their closest phylogenetic relatives. Sequences that differed by less than 3 % were considered to belong to the same phylotype (Stackbrandt and Goebel, 1994); and each phylotype was represented by a sequence type. Editing and cleaning of sequence chromatograms was performed using Finch TV Version 1.4.0 (www.geospiza.com). The DNAMAN version 4.0 software Lynnon Biosoft (Department of Microbiology, University of Cape Town) was employed for sequence alignment and construction of the phylogenetic trees using the neighbor-joining algorithm (Saitou and Nei, 1987). Only the alignment without ambiguous regions was used for phylogenetic analyses. Bootstrap analysis value of 1,000 was applied to assign confidence levels to the nodes in the trees.

5.2.2 Scanning electron microscopy

Scanning electron microscopy (SEM) is a very useful tool for the visualisation and interpretation of certain bacteria features that cannot be appreciated with a light microscope but are important for taxonomic identification. The specimens were collected from both bioreactors (mesophilic and thermophilic). One millilitre of samples containing about 1g of granules was placed in 7ml tubes, rinsed three times (5 minutes each) in distilled water and then fixed in 8 % glutaraldehyde 25 % EM grade (diluted in Ringer's solution pH 7.3) overnight. The samples were dehydrated at 10 minute intervals through 30, 50, 70, 90, 95 and 100 % ethanol, after which they were critical point dried with CO_2, mounted on SEM stubs, coated with gold and studied using JEOL JSM 840 scanning electron microscope equipped with a digital Nikon F301 camera.

5.3 Results and Discussion

The existence of diverse microbial populations within the bacterial granules maintained at different temperatures was verified by the DGGE profiles as shown in Figure 5.1 and 5.2. Each band on the DGGE profile corresponded to a gene fragment of unique 16S rDNA sequences and accordingly represented a specific species in the microbial community. Fifty unique band positions were identified on UV, however only thirteen could be screened by Inqaba, South Africa. Nine of these thirteen bands were common to 45°C reactor (Lane A_2, B_2, C_4, D_7, E_2, F_3, G_2, I_2 and J_3) (Figure 5.1) and the other four bands to 70°C reactor (Lane:

D_6, E_6, I_6 and J_2) (Figure 5.2). When the temperature of the reactor was increased from 45°C to 70°C, the number for 16s rDNA PCR amplicon bands decreased from nine to four. This can be attributed to the selective force acting upon the microbial community, and this is because microbial respiratory activity or general physiology was optimally adapted to the operating temperature. DGGE profiles clearly showed the complexity of the microbial communities decreasing substantially at higher temperatures (Figure 5.2). Generally, microbial diversity decreases with the increase of both sludge pre-treatment temperature (Baghchehsaraee *et al*., 2008; Li *et al*., 2011) and operation temperature (Karadag and Puhakka, 2010; Li *et al*., 2011), but increases with pH (Fang and Liu, 2002; Fang *et al*., 2006; Li *et al*., 2011). LaPara *et al*., 2000 demonstrated that some mesophilic microbes might have more difficulty than thermophilic microbes to maintain their cellular integrity when bioreactor ran at high temperatures (70°C). The banding patterns on DGGE gels do not necessarily represent the composition of the entire microbial community, but does indicate the dominant phylotypes in the sample. Ecological theory suggests that (a) different species will be selected at different temperatures and (b) extreme environmental conditions (e.g. high temperatures) will reduce diversity (Begon *et al*., 1990).

A_2, B_2, C_4, D_7, E_2 F_3,G_2, H I_2 J_3, K

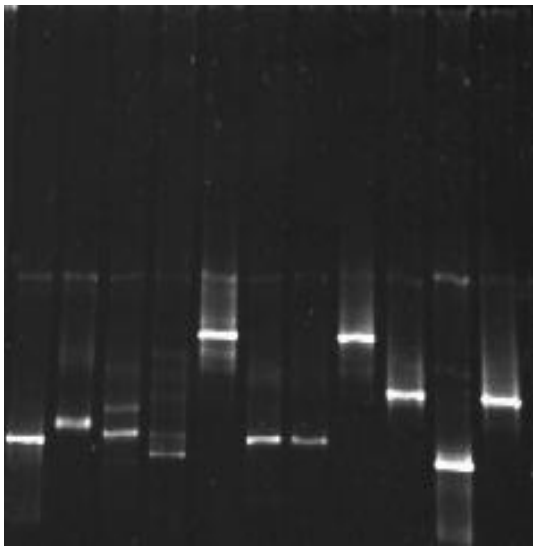

Figure 5.1 DGGE profiles of partial 16S rDNA fragments amplified from the hydrogen-producing reactor operated at 45°C: Lane A_2, B_2, C_4, D_7, E_2, F_3, G_2, I_2 and J_3.

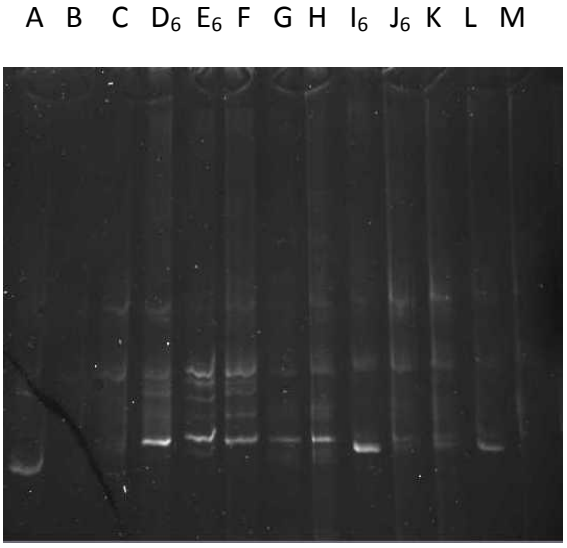

Figure 5.2 DGGE profiles of partial 16S rDNA fragments amplified from the hydrogen-producing reactor operated at 70°C. Lane D_6, E_6, I_6 and J_2.

Sequence similarity searches were performed using the Basic local alignment search tool (BLAST) program to search NCBI sequence database (http://www.ncbi.nlm.nih.gov/BLAST/). Results of their closest relative are shown in table 5.1. To better visualise the relationships among them, a phylogenic tree of sequenced DGGE bands was constructed, (Figure 5.3). It can be seen that the 16S rDNA sequences of the bioreactor samples were closely related with clostridia (*Clostridium thermobutyricum Clostridium butyricum* and *Clostridium thermopalmarium*) and other species such as *Klebsiella* species and *Pseudomonas aeruginosa* were also found.

Table 5.1 shows the highest percentage and closest related species for each DGGE from the NCBI database using BLAST.

DGGE band	Accession number	Closest identified phylogenetic relationship	Percentage similarity
A_1(D6, E6, I6, J2)	NR_028934 and NR_026327	*Clostridium thermobutyricum* and *Clostridium thermopalmarium*	99%
B_1 (A_2, B_2, C_4, D_7, F_3, G_2 J_3)	GU227152	*Clostridium butyricum* (C14-3)	95%
B_2 (E_2, I_2)	HQ200406	*Klebsiella pneumoniae* (K) *Pseudomonas aeruginosa*	99%

Most researches describe *Clostridium* species as rods forming endospore, with strictly anaerobic growth, absence of dissimilatory sulphate reduction, positive Gram type, and responsible for the anaerobic bioproduction of H_2 (Fang *et al*., 2006; Lee *et al*., 2007). These bacteria can be located in marine sediments at various depths of the ocean, thermophilic environments and in the intestinal tract of animals (Lee *et al*., 2007). *Clostridium* species is known for evolving H_2 during anaerobic fermentation from different substrates (Fang *et al*., 2002; Karube *et al*., 1982; Iyer *et al*., 2004; Wang *et al*., 2008b). As an H_2 producer, *Clostridium* was believed to impart several significant advantages, such as production of organic acids by traditional fermentation (mainly butyric acid) (Lin *et al*., 2008) and alcohols from carbohydrates and peptones (Ueno *et al*., 2001b), metabolise carbohydrates, alcohols, amino acids, purines, steroids and other compounds.

Clostridium thermobutyricum is a thermophile and can be differentiated from mesophilic clostridia by its ability to grow at temperatures above 55°C (Wiegel *et al*., 1979) while *Clostridium thermopalmarium* is a moderate thermophile (Lawson Anani Soh *et al*., 1991). *Clostridium butyricum* are mesophilic (Berckers *et al*., 2010). However, in this study, it was found that *Clostridium thermopalmarium* could grow at 70ºC.

Klebsiella pneumoniae and *Pseudomonas aeruginosa* are mesophilic, gram-negative, and facultative anaerobes (Chen *et al*., 2006). These bacteria are recognised also in the research as to generate H_2 gas (Lin *et al*., 2008). Solomon *et al*. (1995) observed cell growth of

Klebsiella pneumoniae in anaerobic batch fed with glycerol (40 g/L), basal medium plus yeast extract (1 g/L), pH 7.0 at 33ºC. Chen *et al.* (2006) studied the glucose metabolism of *Klebsiella pneumoniae* cells under anaerobic conditions and observed that H_2 generation was mainly associated with nitrogenase activity.

Clostridium species is extremely sensitive to O_2. Their H_2 producing abilities are inhibited by a tiny amount of O_2 in the reactor. However, the presence of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* in the bioreactor might focus on the positive side of promoting H_2 production by maintaining a strict anaerobic condition suitable for the surviving of major H_2 producers (*clostridium*) (Chapter 3 and 4). This syntrophic co-culture system involved aerobic and anaerobic procedures; ability of O_2 consumption for creating the anaerobic environment is needed by the partner microbe (Chang *et al*., 2008).

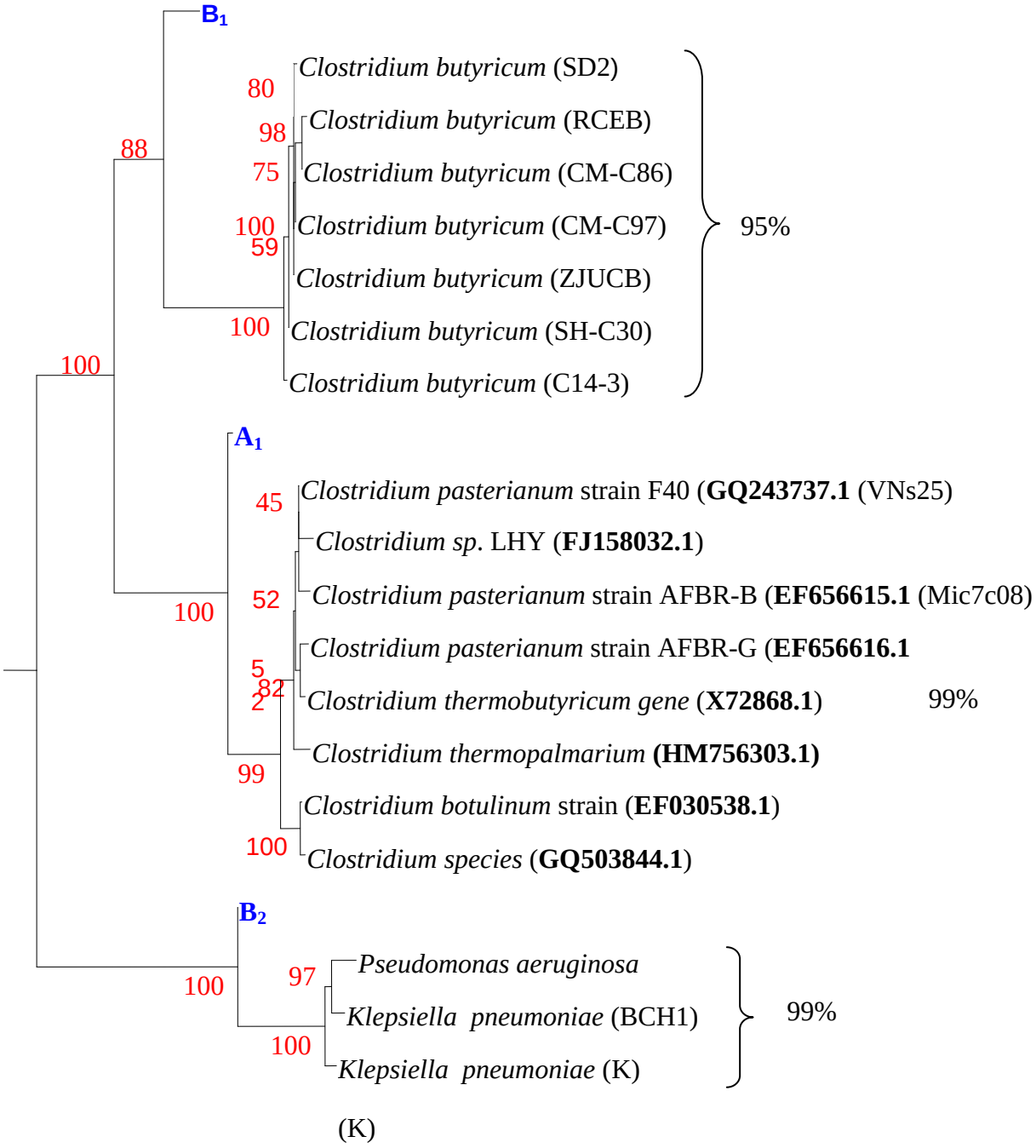

Figure 5.3 Neighbour-joining phylogenetic tree based on partial 16S rDNA sequences derived from mesophilic and thermophilic AFGB DGGE bands. The tree was constructed by using DNAMAN software. Bootstrap values indicate areas of stable tree topology based on 1000 bootstrap resamplings. Nucleotide sequence accession numbers or strain names and similarity (%) are indicated.

A close examination by SEM revealed that microbial morphology in the thermophilic bioreactor consisted predominantly of long, rod-shape bacteria. The bacteria were strongly attached to each other by extracellular polymeric substances (EPSs). The EPS play an important role in the promotion of the initial attachment of cells to solid surfaces; formation and maintenance of microcolony and mature biofilm structure; and enhanced biofilm resistance to environmental stress and disinfectants. In some cases, EPS matrix also enables the bacteria to capture nutrients (Czaczyk and Myszka, 2007). According to Dolfing 1987, the production of EPSs by some microorganisms under certain conditions is considered as the factor responsible for the phenomenon of anaerobic granulation (Figure 5.4)

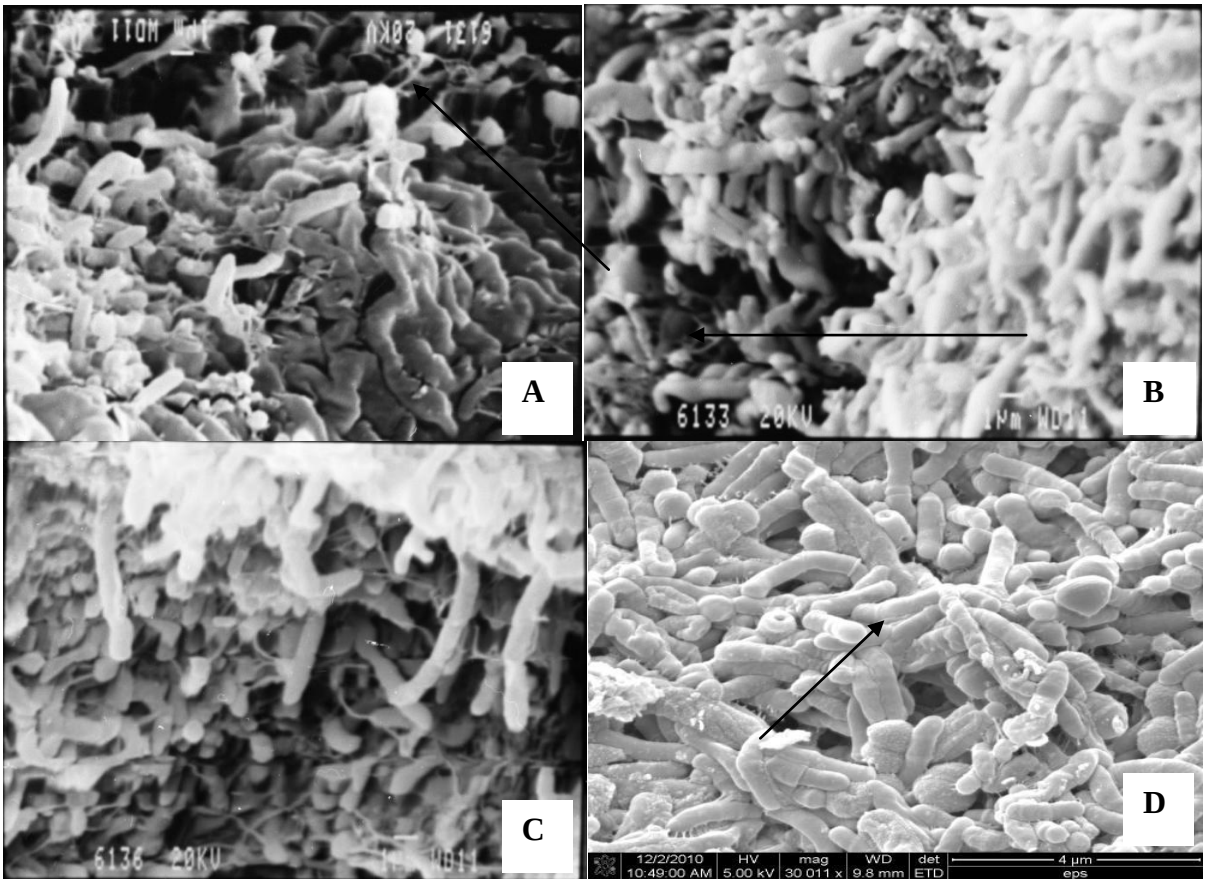

Figure 5.4 SEM images for (A and B) showing interior porous structure. C and D show predominant bacterial morphology of the H_2 producing granules, the arrow shows extracellular polymers for bacterial attachment.

- **Physico characteristics of the granules**

The characteristics of the formed granules observed in this study were the following: Compact spherical granules mainly observed in thermophilic bioreactor and elliptical and

irregular shapes were observed in mesophilic bioreactor. Their diameter varied from 1.3 to 2.7 mm as shown in Figure 5.5 taken with light dissecting microscope.

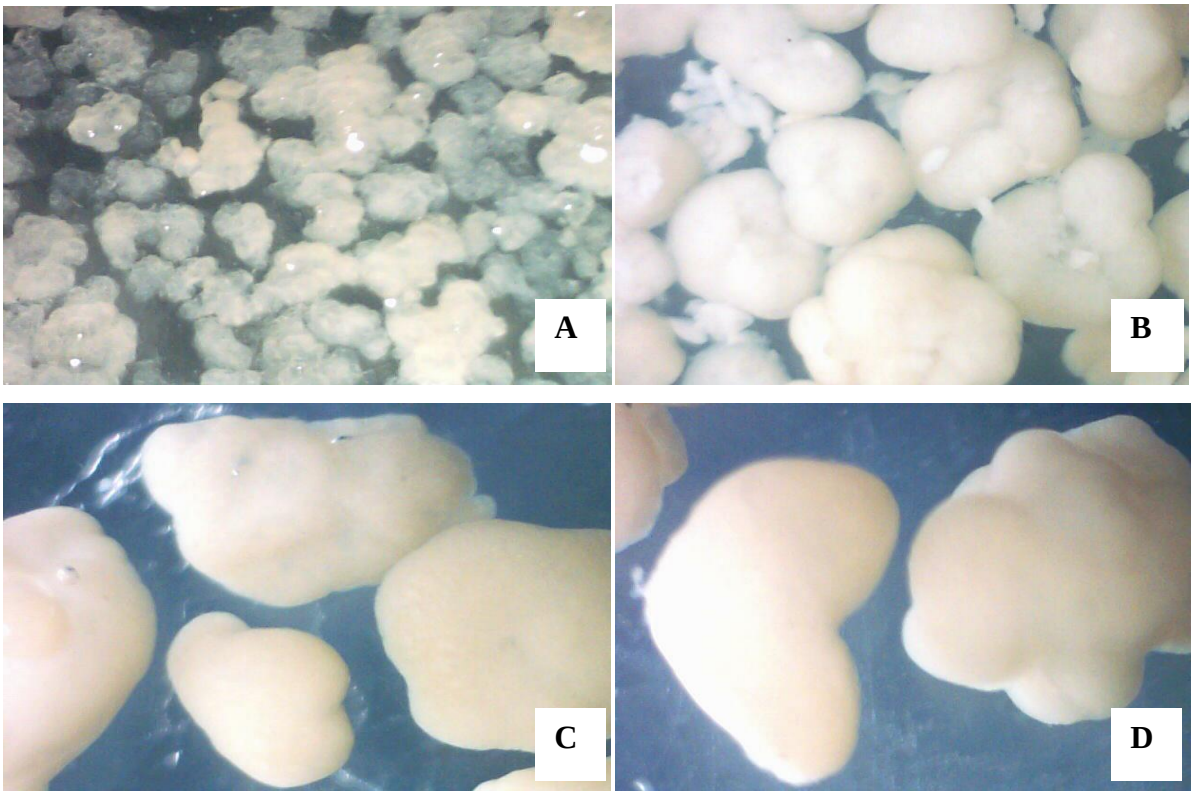

Figure 5.5 Granules formation during anaerobic biohydrogen, using a light dissecting microscope. A) Starting: Bacterial flocs B, C, D) matured granules

The DNA-based PCR-DGGE profile failed to identify all the H_2 producing bacteria community in the bioreactor. It might be due to the fact that the quantity of some bacteria strain were not enough to be detected by PCR-DGGE analysis especially those that have low band intensity. This suggests that bacterial populations present in the bioreactor were in small quantities and were repressed by other dominant strains. Therefore, microbial community analysis by DNA-based 16S rDNA gene DGGE alone was insufficient to identify bacteria community.

5.4 Conclusion

In conclusion, DNA was extracted, with respect to the increase and decrease in the product concentrations, in order to investigate changes in bacterial community structure. Analyses of the DGGE profiles demonstrated that the bacterial community shifted in the bioreactor due to the increase of temperature during the process. At the beginning of the process, the diversity of mesophilic bacteria is very high and even some types of pathogens are present. During the

operation, these bacteria were reduced, but diversity of thermophilic and thermotolerant bacteria were increased. This suggested that temperature conditions can have an influence in the increase or decrease of H_2 productivity (chapters 3 and 4). Compared to mesophilic fermentation conditions, thermophilic digestion is four times more intense, has higher volatile suspended solids (VSS) removal efficiency and yields more biogas. The only disadvantage of thermophilic stabilisation is that more energy is used for heating the bioreactor (Vindis *et al*., 2009). Based on the findings of the present study, the use of undefined mixed cultures for fermentative H_2 production is encouraged. Future attempts to analyse the total biodiversity of mixed microbial communities should therefore complement these PCR-DGGE based techniques with cloning.

Chapter 6

Thermodynamic analysis of constraints on dark anaerobic hydrogen production

6.1 Introduction

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; Das, 2009; 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;). Attempts to improve both the productivity and yield of biohydrogen generation in dark anaerobic processes appear to have reached the point of diminishing returns (Rittmann 2008). Under most bioreactor design and operation conditions, the maximum possible H_2 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 glucose. In this reaction, the 24 electron equivalents (e^- eq) of glucose, 8 e^- eq end up in H_2 and the remaining 16 e^- eq end up in acetate. In dark fermentation, HY appears to be *stuck* at 4 mol H_2/mol glucose (Rittmann 2008). Theoretically, acetate could be further oxidised 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 that would facilitate the complete oxidation of glucose to 12 H_2 remains an interesting, but controversial consideration (Hallenbeck 2009; Hallenbeck and Gosh 2009). It is the general scientific consensus that formidable hurdles remain to be overcome before the complete oxidation of glucose to hydrogen at high rates in a multiple stage process can be realised in practice (Hallenbeck and Gosh 2009).

This judgment is consistent with the current body of published knowledge on dark anaerobic biohydrogen. In addition, the current body of knowledge is sufficiently detailed and comprehensive to support the development of a reliable expert decision making system that could be used to logically generate a rational *yes* or *no* verdict on the viability of biohydrogen

as an alternative biofuel. Also, application of such an expert system would assist in the making of the most rational decision in the selection of a bioreactor system for biohydrogen production. For example, knowing that a 5 kW fuel cell, which has the capacity to power a house, requires a H_2 supply rate of 2 900 L/h (Levin *et al*., 2004), allows for rational *yes* or *no* judgments to be made on the viability of H_2 rates for specific bioreactor system. Volumetric HPs ranging from 7.3 L H_2/(L.h) to 9.3 L H_2/(L.h) have been achieved for mesophilic fluidized granular bed bioreactors (Lee *et al*., 2004; Lee *et al*., 2006). Assuming that the high HP of such bioprocess could be maintained with scale up, then a 300 L fluidized granular bed bioreactor would be able to power a 5 kW fuel cell. In a counter example, a 400 L thermophilic trickle bed bioreactor with an HP of 22 mmol H_2/ (L.h) was reported to produce 100 mol H_2/day (van Groenestijn *et al*., 2009). Given that a 5 kW fuel cell requires a minimum H_2 supply of 120 mol H_2/h, it would be necessary to use an 11.5 m^3 thermophilic trickling bed bioreactor to power a single 5 kW fuel cell. This is 38.3 times larger than the mesophilic granular bed bioreactor. This kind of analysis provides rational grounds for accepting one process and rejecting the other. In the practical business of technology development, application of expert systems provides rational grounds for the rejection of a particular bioprocess. Expert systems are critical for decision-making in technology development. In any scientific enterprise, the business of theory testing and hypothesis falsification is taken as a measure of progress. If a particular formulation of a scientific theory is used as the reason for pursuing a given technological objective, and this theory becomes subsequently falsified, then in all likelihood, the technological objectives need to be rejected as practically unrealizable. In evaluating the success of bioreactor development task, the relevant hypothesis to be tested may be stated as follows: There exists a thermophilic bioprocess in which high HYs and high HPs can be simultaneously achieved without the concomitant occurrence of a system energy deficit and which at the same generates sufficient net chemical work for the powering of a 5 kW fuel cell. This hypothesis was referred to as the Bioreactor Energy Balance Hypothesis or the BEB Hypothesis. This research proposes that if this hypothesis is falsified, then the thermophilic bioprocess developed designed is this study must be rejected as energetically non-viable. Also, by logical extension, no other viable high rate process exists. From the existing body of published knowledge regarding biohydrogen production together with the laws of thermodynamics, all the necessary and sufficient conditions that are most likely to facilitate the simultaneous achievement of high HYs and high HPs can be explicitly stated as follows:

1. High microbial volumetric biomass density. This would require bacterial granulation.
2. High rates of microbial biomass retention within the bioreactor. This would require bacterial granules with good settling properties.
3. Low hydraulic retention times or high dilution rates.
4. High organic substrate loading rates.
5. Maintenance of the lowest possible partial H_2 pressure within the fluidized bacterial granular bed.
6. Efficient effluent gas-disengagement.
7. High rates of de-gassed effluent recycling through the fluidized granular bed.
8. Maintenance of thermophilic temperatures within the bioreactor increase HY.
9. Minimisation of dead volume within the bioreactor system.

Conditions 1 to 9 were the premises of the working hypothesis for this investigation undertaken in this study. Given the realisation of these nine conditions, the simultaneous achievement of high HYs and high HPs would be the first prediction of the BEB Hypothesis. The second prediction is that the balance energy of the overall process would allow for the production of sufficient energy for net useful work to be done on a 5 kW fuel cell by the bioreactor system.

- ## Theoretical considerations

Any measurement for quantifying the performance of a given bioprocess with respect to a predefined goal should be defined as a performance achievement quantifying *metric*. Generally, metrics deal with the theory of measurement. In the case of bioprocesses for H_2 generation, the two metrics that appear regularly in the literature are hydrogen yield (mol H_2 / mol glucose) and the volumetric hydrogen productivity (L H_2/ (L.h) or mmol H_2/ (L.h)). However, while both HY and HP are useful performance measurements, they do not provide sufficient information on the overall viability of a biohydrogen generation process. Additional metrics are necessary for evaluating the viability of a biohydrogen generating process with respect to its particular HY and HP values. An important metric was the size or volume of the bioreactor required to supply sufficient H_2 to power a 5 kW fuel cell. A 5 kW fuel cell was chosen because it represented the minimum fuel cell power output capacity necessary for a house requiring a maximum of 55 kW per day (Levin *et al*., 2004). The minimum H_2 flow rate necessary for powering a 5 kW fuel cell was 120 mol H_2 /h (Levin *et al*., 2004). Using this information, the minimum viable bioreactor HP can be calculated. In

this case, the minimum HP per m^3 would be 120 mol H_2 / (m^3 .h) which corresponds to 120 mmol H_2/ (L.h). Therefore, in terms of a fuel cell - bioreactor volume metric (abbreviated FCM), the critical threshold for a bioreactor process to be viable would be an HP not less than 120 mmol H_2/ (L.h) irrespective of HY. Should a bioreactor's FCM be less than 120 mmol H_2/ (L.h), then the bioprocess is not practically viable and should be rejected irrespective of its HY. At mesophilic temperature (45^oC), an HP of 300 mmol H_2 / (L.h) has been achieved for anaerobic fluidized granular bed bioreactor (Lee *et al*., 2004). With an HP of 300 mmol H_2 / (L.h), the bioreactor volume required for generating sufficient H_2 to power a 5 kW fuel cell was 400 L or 0.4 m^3. In this example the bioreactor's HP exceeds the minimum FCM of 120 mmol H_2/ (L.h) by a factor of 2.5. In later sections, this factor was called *z*.

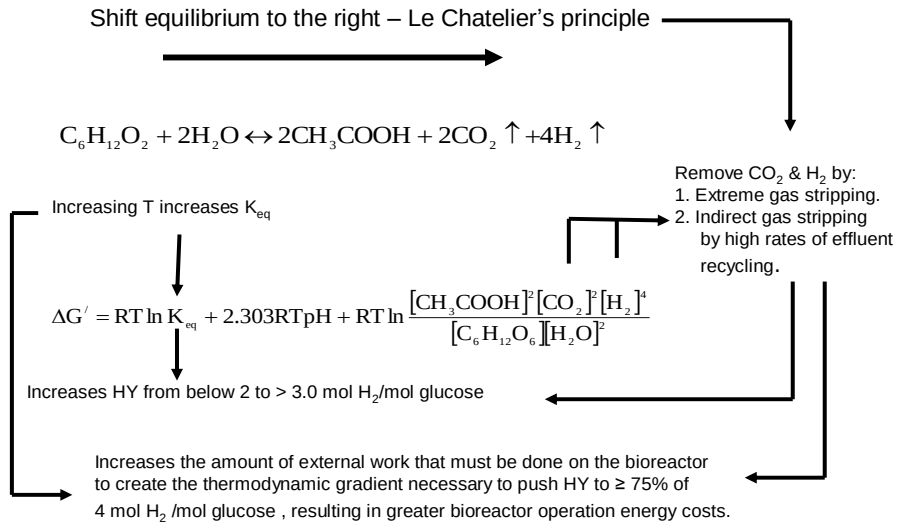

Figure 6.1 Gibb's free energy balance corresponding to bioreactor's HY**.**

What is the significance of HY in relation to FCM? Clearly, if the HP is below the FCM, then the issue of HY is irrelevant. Simply stated, the process has failed irrespective of the HY. HY is a problematical metric. The theoretical maximum value for HY is 4 mol H_2 / mol glucose. With respect to evaluating bioreactor performance, the critical threshold for the HY metric can for practical purposes, be set at 75% of the theoretical maximum or 3 mol H_2 / mol glucose. It is of critical importance to note that in practice HY values equal to or exceeding 3 mol H_2 / mol glucose are usually only attained in situations where HP is several orders of

magnitude below the critical FCM limit of 120 mmol H_2/ (L.h). Conditions that favour high HYs can be summarised as follows: possibly thermophilic temperatures, low substrate loading rates, low dilution rates, low H_2 partial pressures and low bacterial biomass densities. In addition, H_2 gas stripping by sparging with N_2 is usually a necessary precondition for the achievement of HYs equal to or greater than 3 mol H_2 / mol glucose. In all the instances where high HPs have been achieved, the following bioreactor operational conditions have prevailed: high substrate loading rates, high dilution rates, and high bacterial biomass densities. Operational conditions that favour high HPs also promote the maintenance of high H_2 partial pressures within the bioreactor environment. High H_2 partial pressures within the bioreactor environment do not favour 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 favour 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 H2 / mol glucose (Chong *et al*., 2009; Das 2009; Davila-Vazquez *et al*., 2008; Wang and Wan 2009). A possible reason for this is that in reality, HY is also an index or metric of the additional external work that needs to be applied to a bioreactor system in order to force the equilibrium of the reaction in Figure 6.1 to shift further to the right hand side.

6.2 Results of the modified fluidized bed bioreactor

Following the successful induction of bacterial granulation at thermophilic temperatures ranging from 45^{o}C to 70^{o}C, the HP and HY results that were obtained were similar to those reported by Lee *et al*., (2006) for a mesophilic bioreactor. Hence the works of Lee *et al*., (2006) and Zhang *et al*., (2008a) were reproduced at the thermophilic level in this research study. However, in order to increase HY, it was discovered that the bioreactor system and operation had to be modified in significant ways. Firstly, the total volume of the bioreactor system had to be reduced substantially (Chapter 4). The total system volumes of the first bioreactor experiments was 10.5L (Chapter 3), however, the total system volume was reduced in subsequent experiments to 5.5 L as it became clear that the V/R quotient had to be reduced considerably in order to simultaneously increase both HY and HP. It was observed that the larger bioreactor system, for the highest possible degassed effluent recycle rates consistently produced maximum HYs not much than 2 mol H_2/mol glucose reduction of the system volume resulted in a dramatic increase in the HYs. All these modifications that resulted in an increase in the HY were consistent with the energy and work balance scheme

depicted in (Figure 6.1). Removal of the additional volume while increasing HY also resulted in higher levels of sucrose washout. Importantly, the experiments supported the proposal that high HYs could be achieved through the combined effects of reducing bioreactor volume and increasing effluent recycle rates.

6.2.1 Analysis of the bioreactor energy balance

Given that this study showed that high HYs and high HPs could be simultaneously achieved, the next question that needs to be answered is whether the system actually produces net useful work. To answer this question, the energy input and output balance of the bioreactor systems used in this study were analysed (Figure 6.2).

Radiant energy losses: Radiant energy emitted from the bioreactor and gas-disengager surface can be calculated from the Stephan-Boltzmann law (Equation 6.1):

$$R = \varepsilon\sigma T^4 \qquad \text{Equation 6.1}$$

where R is the emitted flux density (W/m^2), T is degrees Kelvin, ε is the emissivity of the bioreactor surface (0.9) and σ is the Stephan-Boltzmann constant (5.67 x 10^{-8} W m^{-2} K^{-4}). Radiant energy emitted from the bioreactor with a surface temperature of 65^{o}C is 667.2 W m^2.

Convective heat losses: Free convective heat transport from the surface of the bioreactor can be calculated from equation 6.2:

$$\frac{dH}{dt} = \frac{\rho_a C_a (T_s - T_a)}{R_H} \qquad \text{Equation 6.2}$$

Where H is the free convective heat flux from the surface of the bioreactor and gas-disengager, ρ_a is the density of air (kg m^{-3}), C_a is the specific capacity of air (1.01 kJ kg^{-1} ^{o}C^{-1}), T_s is the surface temperature of the bioreactor, T_a is ambient air temperature, R_H is the sensible heat transfer resistance (s m^{-1}). Convective heat flux from the bioreactor with a surface temperature of 65^{o}C is 217.3 W m^{-2} . The heat transfer resistance in free convective heat flux is given by equation 6.3:

$$R_H = \frac{d}{0.54 D_H (Gr\,\mathrm{Pr})^{0.25}} \qquad \text{Equation 6.3}$$

where *d* is the characteristic dimension of the bioreactor, D_H is the thermal diffusivity, *Gr* is the Grashof number and *Pr* is the Prandtl number. The Grashof number is computed as follows (Equation 6.4):

$$Gr = \frac{agd^3(T_s - T_a)}{\nu^2} \qquad \text{Equation 6.4}$$

where *a* is the coefficient of thermal expansion for fluid (a = 1/273 for air), g is the gravitational acceleration (9.8 m s^{-2}), ν is the kinematic viscosity.

The Prandtl number is computed as follows (Equation 6.5):

$$\Pr = \frac{\nu}{D_H} \qquad \text{Equation 6.5}$$

Influent heating: Energy (*Q*) required to heat nutrient influent from 25 ^oC to 65 ^oC depends on the influent rate (*F*) in L s^{-1} and is calculated as follows (Equation 6.6):

$$\frac{dQ}{dt} = FC_w(T_s - T_{a)} \qquad \text{Equation 6.6}$$

Where C_w is the specific heat of water (4.19 kJ kg^{-1} $^oC^{-1}$). Energy demand for influent heating depends on the HRT. Highest HYs and HPs for a 5.0 L bioreactor were achieved for influent flow rates of 0.21 L min^{-1} and the energy required to heat the influent to 65 ^oC was 660 W. There is therefore an energy loss of 660 W, however with heat recovery by allowing heat exchange in a counter current fashion between the outgoing effluent stream and the incoming influent stream then the heat loss can be substantially reduced. Assuming the incoming influent stream will be 45 ^oC as a consequence of heat recovery, and then the energy loss will be reduced to 293 W.

Energy used for effluent recycling: The mechanical energy (*M*) required for the work of influent pumping and effluent recycling was 380 W.

6.2.2 Analysis of the BEB Hypothesis

In order to shift the equilibrium of the above reaction further to the right, additional work in the form of heat energy and mechanical energy needs to be supplied to the bioreactor system (Figures 6.1). An additional metric based on the bioreactor's net energy balance needs to be formulated to assess the practical viability of the bioreactor system operating under conditions that favour the achievement of both high HYs and high HPs. Table 6.1 gives a breakdown of the energy inputs and the energy outputs calculated for the bioreactor system in Chapter 3. Heating of the influent results in the highest energy cost, almost double the energy cost required for high rates of effluent recycling. It is interesting to note that more energy is required for influent heating from $25^{o}C$ to $65^{o}C$ than for recycling of effluent against gravity. The external work done on the system in order to increase HY results in the bioreactor system having a net energy deficit (Table 6.1). A very important goal in the operation of the bioreactor is heat recovery. Heat can be recovered by using the exiting effluent stream to heat the incoming influent steam in a counter current heat exchange. The heat exchange will be proportional to the temperature differences between the effluent stream and the influent stream. The influent stream will be at ambient temperature whereas the effluent stream will be at the selected thermophilic temperature. In short, the heat recovery is vital for the positive bioreactor energy balance sum of energy inputs, exceeds bioreactor energy output in the form of hydrogen energy (Table 6.1). If the bioreactor is effectively insulated, its surface temperature should be close to ambient temperature. The energy losses due to radiation and sensible or convective heat transfers could be reduced further and thereby increase net positive energy production by the bioreactor.

Table 6.1 Biohydrogen bioreactor energy balance for a mesophilic and thermophilic bioreactor. Total bioreactor volume was 5.0 L and total system volume was 7.5L.

Energy inputs/outputs	**Bioreactor energy balance**
1. With no heat recovery	kW
Sensible heat convection (*H*)	0.105
Radiant emittance (*R*)	0.215
Heating of influent (*Q*)	0.660
Mechanical energy (*M*)	0.380
Total energy input	1.360
Hydrogen energy output (*HE*)	1.212
Net useful energy production (*W*)	-0.148
2. With heat recovery	kW
Sensible heat convection (*H*)	0.105
Radiant emittance (*R*)	0.215
Heating of influent (*Q*)	0.293
Mechanical energy (*M*)	0.380
Total energy input	0.963
Hydrogen energy output (*HE*)	1.212
Net useful energy production (*W*)	+0.219

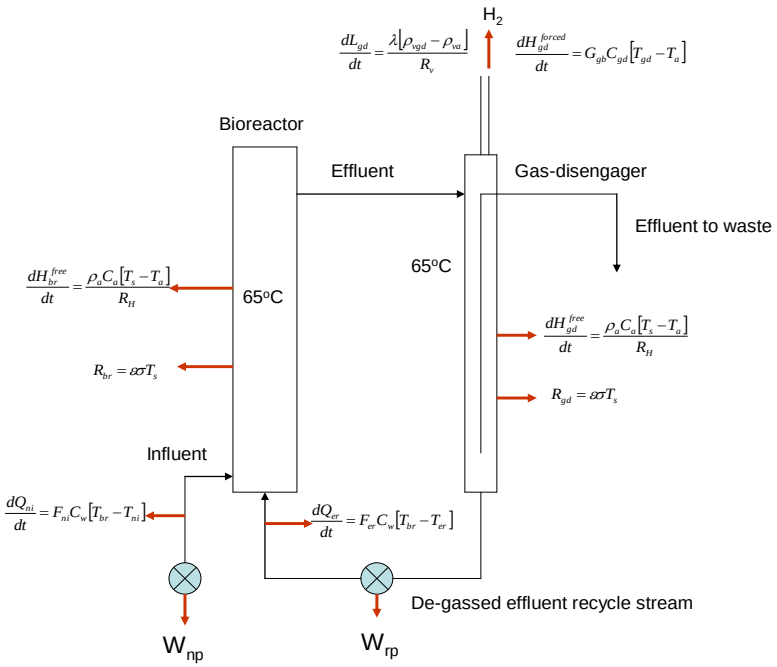

Figure 6 2 Bioreactor energy input-output balances**.**

6.2.3 The second analysis of the BEB hypothesis

In this study, it also became clear that an expert system should be developed for any biohydrogen commercialisation scale-up process. In the development of the modified fluidized bed bioreactors, a set of critical factors that either increase or reduce the viability of a H_2 generating bioprocess were identified. These critical factors were formulated into a set of premises. Such set of premises can readily be used in developing a logical structure for a biohydrogen expert decision-making system. The set of premises deal with all the factors and conditions that affect the energy balance or thermodynamics of the H_2-generating bioprocess. The thermodynamics or energy balance of bioprocess supplies the information necessary for generating a logical *yes* or *no* decision regarding the viability of a biohydrogen process at any stage in its development. How the expert system works was outlined in a simplified evaluation of the viability of a commercial scale bioprocess for H_2 generation. Analysis of the energy balance in kWh for a scaled-up model commercial bioreactor with a diameter of 2.0 m, height of 16 m (aspect ratio = 8.0), working volume of 50 000 L and surface area of 100 m^2 was used as the example to assess the viability or non-viability of the bioprocess. It is important to note that all the premises used in this analysis are empirically

valid or can in principle be empirically validated. The logical basis of the expert system rests on some form of bioreactor energy balance relationship (Equation 6.7):

$$W = HE - M_i - M_{er} - Q - R - H_{free} - H_{forced} - \lambda E \qquad \text{Equation 6.7}$$

W is the net work done. *HE* is the H_2 energy produced by the process. If the process is viable, then net useful work will be produced by the system in the form of H_2 energy. If the sum of energy inputs exceeds the energy output in the form of *HE*, then no useful net work is produced by the system. The expert system can be applied as an Excel spreadsheet. The value of *W* would be the output of the expert system. The expert decision will depend on the value of *W*. If *W* has positive sign, the bioprocess has all the attributes for viability. If *W* is negative, then the bioprocess must be rejected as non-viable and the BEB hypothesis is falsified.

The set of premises used in the formulation of the expert system are:

Premise N^o 1: In order to lower the H_2 partial pressure within the bulk fluid phase in which the granules are suspended and thereby increase the thermodynamic gradient driving the H_2 generating reaction (Figure 6.1), recycling of de-gassed effluent above a critical flow velocity is essential for the achievement of high HYs and high HPs. Shifting the equilibrium of the H_2 generation reaction to the right (Figure 6.1) involves work equal to M_{er} being done on the bioreactor. Energy required for effluent recycling (M_{er}) can be computed as follows (Equation 6.8):

$$M_{er} = \frac{1000 F_{er} \pi r^2 g h}{PE \cdot EME} \qquad \text{Equation 6.8}$$

where effluent recycle rate (F_{er}) = 0.0116 m s^{-1}, *PE* = 0.70 is the dimensionless pumping efficiency, *EME* = 0.88 is the dimensionless electrical motor efficiency, *r* is the radius of the bioreactor and *h* is the bioreactor height. The specific volumetric hydraulic power required to operate the bioreactor would then be 187.3 kW m^{-3}. The 5 L bench scale prototype had an empirically verified specific volumetric power of 0.380 kW/5 L = 0.076 kW L^{-1} which would be equivalent to 76 kW m^{-3}. It is assumed that the specific volumetric power increases as per eq n with scale up from 5 L to 50 000 L would not be greater than 2.46 with respect to the empirically verifiable value used for the energy balance of bench scale prototype (Table 6.2).

Premise N^{o} 2: Effective gas-disengagement of the effluent is essential not only for efficient H_2 recovery, but also for de-gassing the effluent prior to being recycled. Effluent gas-disengagement also results in heat loss as a consequence of forced convection (H_{forced}) and latent heat (λE). Heat loss from the gas-disengager results in not less than a 10% decline in bioreactor effluent temperature (6.5^{o}C).

Premise N^{o} 3: High dilution rates or low hydraulic retention times increase HP and also augment the effect of effluent recycling in the removal of H_2 from the bulk fluid phase in which the granules are suspended. To achieve an optimal HRT of 2.78 h for the model bioreactor, the influent flow rate (F_{ir}) should have a magnitude in the order of 50 L s^{-1}. The work arising from head loss or energy consumed as a result of pumping nutrient influent into the base of the bioreactor against fluid head of *h* can be computed as follows (Equation 6.9):

$$M_{ir} = \frac{F_{ir} g h}{PE \cdot EME} \qquad \text{Equation 6.9}$$

M_{ir} = 12.74 kWh

Premise N^{o} 4: Energy inputs (8380 kWh) are required for influent heating from 25^{o}C to 65 ^{o}C at a rate of 50 L s^{-1} (C_w = 4.19 kJ kg^{-1} ^{o}C^{-1}) and the energy required depends on the HRT. Energy is also required for re-heating (992 kWh) the recycled effluent (Δ6.5^{o}C). However, with heat recovery through counter-current heat exchange between effluent and influent streams, the heat cost falls to 4190 kWh.

Premise N^{o} 5: HP should be equal or greater than but never less than the FCM threshold value of 2 900 L/ (m^3 .h), thus ideally, HP $\geq$ z (2 900 L/ (m^3 .h)), where z $\geq$ 1. If z $\leq$ 1, then the bioprocess fails the viability test and should be rejected.

Premise N^{o} 6: Bacterial granulation increases microbial biomass retention in the bioreactor by reducing biomass washout. HP $\geq$ z (2 900 L/ (m^3 .h)) depends on microbial biomass density within the bioreactor. High microbial biomass densities can only be maintained in the bioreactor under conditions of low HRT and high effluent recycling rates if the microbial biomass is organised into granules with good settling properties. Bacterial biomass in the form of granules should fall within the range of 30 to 40 g VSS L^{-1}(Lee *et al*., 2005). Bioprocesses that are not based on granulation will in all likelihood, have a z $\leq$ 1, and will thus probably fail to be viable and should be rejected.

Premise N^{o} 7: Achievement of HPs ≥ z (2 900 L/ (m^3 .h)) will require specific bacterial hydrogen production rates not less than 0.2 L H_2/ (g VSS.h). Assuming a biomass density of 35 g VSS L^{-1}, the total H_2 generation capacity of a 50 m^3 bioreactor should readily reach 350 000 L h^{-1} which corresponds to an HP of 7 000 L/(m^3 .h) and z will equal 2.41.

Premise N^{o} 8: If HY ≥ 3 3.0 mol H2 / mol glucose, but z ≤ 1, then bioprocess is non-viable and should be rejected.

Premise N^{o} 9: With a H_2 energy content = 12 700 J L^{-1}, a total potential H_2 energy output for a 50 m^3 bioreactor should be 5115 kWh for z =1.

It is interesting to note that energy input for heating roughly equals the energy input for effluent recycle (Table 6.2). The process for the model scaled up bioreactor becomes increasingly viable if temperatures are reduced from thermophilic to mesophilic temperatures. The highest reported mesophilic HP equals 9.3 L H_2 / (L.h) at 40 ^{o}C and z = 3 corresponds to 8.7 L H_2 / (L.h). At a mesophilic temperature of 40 ^{o}C, the net changes from -3482 kWh to 10 950 kWh. Clearly, the thermophilic process fails the viability test.

Table 6.2 Energy balance for a scaled up model thermophilic bioreactor.

A. With no heat recovery

Energy inputs/outputs	**Bioreactor energy balance**
	kWh
Sensible heat convection	22
Radiant emittance	67
Heating of influent	8380
Re-heating of recycled effluent	993
Mechanical energy (*M*)	9365
Total energy input	18 827
Hydrogen energy output (*HE*)	
z =1	5115
z =2	10 231
z =3	15 345
Net useful energy production (*W*)	
z =1	-13712
z = 2	-8596
z = 3	-3482

B.With heat recovery

Energy inputs/outputs	**Bioreactor energy balance**
	kWh
Sensible heat convection	22
Radiant emittance	67
Heating of influent	4190
Re-heating of recycled effluent	993
Mechanical energy (*M*)	9365
Total energy input	14 637
Hydrogen energy output (*HE*)	
z =1	5115
z =2	10 231
z =3	15 345
Net useful energy production (*W*)	
z =1	-9522
z = 2	-4406
z = 3	+708

From Table 6.2 B, a positive energy balance becomes possible at HPs that are 3x greater than the benchmark HP and if there is heat recovery from the effluent stream. HPs greater than 3x the benchmark are possible as demonstrated in this study.

6.2.4 Heat exchange and heat recovery possibilities

With regard to heat recovery, the laws of thermodynamics do not allow for any heat flow from a heat sink (T_R) to a heat reservoir (T_1) where $T_R < T_1$. All empirical evidence from these experiments and from the data reported in the literature support the fact that the energy input required for heating of a thermophilic biohydrogen bioreactor system exceeds the energy output in the form of hydrogen chemical energy (Figure 6.3). Heat recovery may indeed occur for downstream processes, but this cannot recover the energy already lost as depicted in (Figure 6.3).

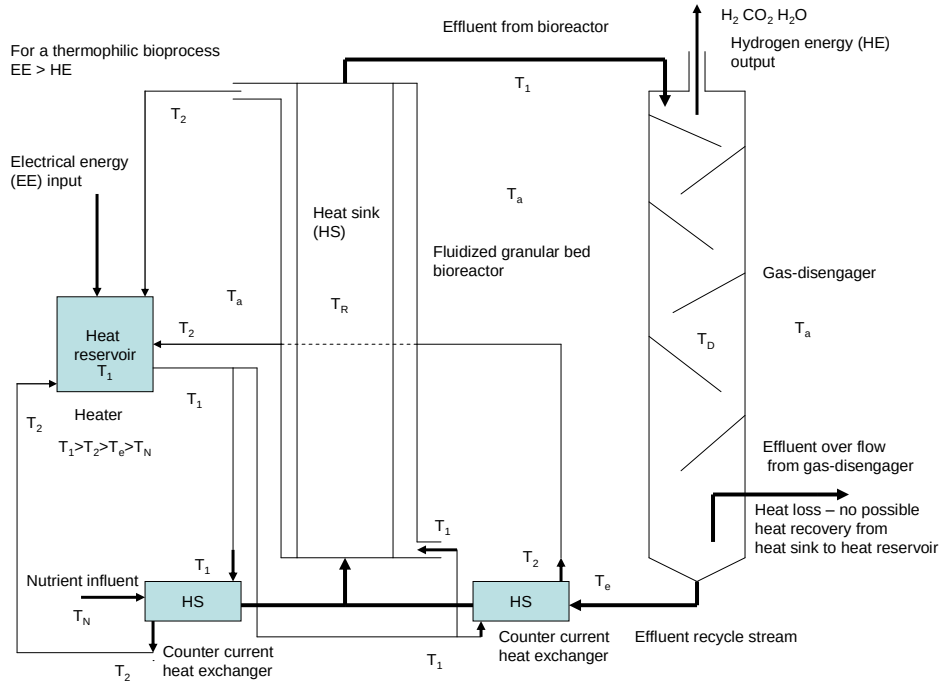

Figure 6 3 Heat exchange and heat flow in the scaled up model biohydrogen system based on a scale of up version of the bioreactor prototype developed in this study (Equation 6.10):

$$\frac{dQ}{dt} = FC_{pc}(T_1 - T_2) = UA\frac{(T_1 - T_{21})}{\ln\frac{(T_1 - T_R)}{(T_2 - T_R)}} = UA(T_1 - T_R) \qquad \text{Equation 6.10}$$

However, as has been shown in Tables 6.1 and 6.2, heat recovery as a consequence of counter current heat exchange between the effluent stream and the influent stream can indeed make the thermophilic biohydrogen production viable with respect to a positive energy balance.

6.2.5 Biohydrogen risk assessment

The results of this research based on the fluidized bed bioreactor experiments and the scaled-up commercial bioreactor model may indeed favour the predictions of the BEB Hypothesis. Thermophilic anaerobic biohydrogen production may indeed be practically viable as a component or first stage system for alternative energy generation. While the focus of this study did not include the second stage, photofermentation bioprocess for hydrogen generation, it requires very little imagination to ensure that for this process to be viable, formidable obstacles have to be overcome.

6.3 Conclusion

In the light of the confirming evidence the BEB hypothesis seems that biohydrogen has potential for the future. It seems possible that a thermophilic bioprocess in which high HYs and high HPs can be simultaneously achieved without the concomitant occurrence of a system energy deficit and which at the same; generate sufficient net chemical work for the powering of a 5 kW fuel cell.

Chapter 7

Conclusion

The research objectives undertaken in this study involved the development of a modified fluidized bacterial granular bed bioreactor prototype. The granules comprised of an undefined anaerobic thermophilic multispecies consortium of bacteria. In order to establish the thermophilic bacterial granules, the bioreactor was operated as a chemostat under increasing dilution rates. This promoted the selection and enrichment of thermophilic granules comprised of a multispecies bacterial consortium. Endo medium which is one of the most basic bacteriological nutrient mediums was used as the nutrient supply in the granule generating chemostat experiments. Sewage was used as the primary inoculum source in the chemostat experiments. Granulation was successfully induced under thermophilic temperatures ranging from 45^{o}C to 70^{o}C within a period ranging from 5 to 14 days. Bioreactor design and operation was modified in order to increase both HY and volumetric HP. It was found that in order to increase both HY and HP it was necessary to implement a number of modifications in bioreactor design and operation. Firstly, the total bioreactor system volume of the bioreactor had to be reduced to 10.5 L which included the 5.0 L bioreactor volume (and with the tubing and gas-disengager making up the remaining 2.5 L) to 5.5 L with a bioreactor volume of 3.0 L. Secondly, the HRT was reduced. Thirdly, the recycling rate of de-gassed effluent through the bioreactor was increased. At an HRT of 1.67 h for a total system volume of 10.5 L (bioreactor volume = 5.0L) and a effluent recycle rate of 3.5 L min^{-1}, an HP of 256 mmol H_2/(L.h) was achieved and a HY of 3.26 mol H_2/ mol glucose was also achieved. Under these operational conditions, sucrose washout also increased, with total consumption of sucrose falling to 84%. In all the thermophilic bioreactor experiments, it was suspected that the biohydrogen generating system would have a net energy deficit, that is, the sum of all the energy inputs would exceed the energy output in the form of H_2 energy. To test whether this was the case, an energy balance analysis was carried out on the bench scale prototype bioreactor. In order to maintain thermophilic temperature at low HRTs and high effluent recycles rates, it was necessary to heat the bioreactors with water from a 25 L heat reservoir (hot water bath). Two 2 kW (16 am)

heater-circulators were found to be necessary to maintain the bioreactor and the gas-disengager at thermophilic temperatures. For the bench scale prototype, the energy calculations confirmed that the sum of energy inputs exceeded the energy outputs in the form of H_2 energy. Furthermore, an energy balance model was developed for a scaled up version of the prototype. Similar results were obtained for the model, energy inputs exceeding energy outputs. This investigation confirmed the prediction that the thermophilic prototype bioreactor system may indeed be practically viable as a bioprocess system for biohydrogen generation. Nor were models of scaled-up versions of the prototype bioreactor viable as bioprocess systems for biohydrogen generation at thermophilic temperatures. In summary, the successful outputs of this study include the following:

1. Induction, development and growth of thermophilic bacterial granules;
2. Development of a modified fluidized bed bioreactor based on bacterial granules;
3. Demonstration that decreasing HRT and increasing effluent recycling rates were key bioreactor operational factors with regard to increasing both HP and HY;
4. Demonstration that a thermophilic bioreactor with high HPs and high HYs was thermodynamically or energetically viable because the energy inputs did not exceed the energy outputs.

Compelling scientific evidence has been presented in this study which demonstrates that a high rate thermophilic bioreactor such as the one developed and tested in this investigation is in fact potentially energetically viable. It is also critical to note that the single most important factor that makes the process non-viable are the heat energy inputs required for the maintenance of thermophilic temperatures and not the energy required for effluent recycling. This is the most critical and far-reaching conclusion reached in this study. It places a huge question mark over the viability of thermophilic dark fermentation processes for biohydrogen generation. After weighing-up all the bioreactor evidence (both empirical and modelling studies) generated from this study and from a thorough review of all recent literature on biohydrogen, there is reasonable evidence that provides unambiguous support for the practical viability of a thermophilic biohydrogen generation bioprocess. The high-lights for this study include:

1. Induction, development and growth of thermophilic bacterial granules;
2. Achievement of high volumetric bacterial biomass densities;
3. Operation of the bioreactor low HRTs and high organic substrate loading rates;
4. Operation of the bioreactor at high effluent recycles rates.

In conclusion, these experiments have reproduced and confirmed all the conditions that are necessary for achieving a high rate thermophilic dark fermentation bioprocess for H_2 generation. Finally, this study has also confirmed that a high rate thermophilic bioreactor will fail all energy balance viability tests.

Chapter 8
References

Adams, C.J., Redmond, M.C. and Valentine, D.L. (2006). Pure-culture growth of fermentative bacteria, facilitated by H removal: Bioenergetics and H production. *Applied and Environmental Microbiology*.**72:** 1079 – 1085.

Agama Energy. (2007). Integrated biogas solution.

Amann, R. I., Ludwig, W. and Schleifer, K.H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiology Revue.* **59:**143-169.

Angenent, L.T., Sung, S. and Raskin, L. (2002) Methanogenic population dynamics during startup of a full-scale anaerobic sequencing batch reactor treating swine waste. *Water Resources*. 36:4648-4654.

Aoyama, K., Uemura, I., Miyake, J. and Asada, Y. (1997). Fermentative metabolism to produce hydrogen gas and organic compounds in a cyanobacterium, Spirulina platensis. *Journal of Fermentation and Bioengineering* **83 :**(1) 17–20.

Arctic Climate Impact Assessment (ACIA). (2004). Impacts of a warming Artic: Artic Climate impact Assessment. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 144 pp.

Baghchehsaraee, B., Nakhla, G., Karamanev, D., Margaritis, A. and Reid, G. (2008). The effect of heat pretreatment temperature on fermentative hydrogen production using mixed cultures. *International Journal of Hydrogen Energy*. **33 (15):** 4064–4073.

Bailey, J.E. and Ollis, D.F. (1986). Biochemical engineering fundamentals, 2nd ed. McGraw-Hill Book Co.: New York.

Balat, M. (2006). Biomass Energy and Biochemical Conversion Processing for Fuels and Chemicals. *Energy Sources, Part A.* **28**:517–525.

Barros, A.R., de Amorim., E. L. C., Reis, C. M.; Shida, G. M. and Silva, E. L. (2010). Biohydrogen production in anaerobic fluidized bed reactors: effect of support material and hydraulic retention time. *International Journal of Hydrogen Energy*. **35:** 3379 – 3388.

Beckers, L., Hiligsmann, S., Hamilton, C., Masset, J. and Thonart, P. (2010). Fermentative hydrogen production by *Clostridium butyricum* CWBI1009 and *Citrobacter freundii* CWBI952 in pure and mixed cultures. *Base*. **2 (14):** 541-548.

Begon, M., Harper, J. L. and Townsend, C. R. (1990). Ecology-Individuals, Populations and Communities, Blackwell Scientific Publication, London, UK, 2nd edition.

Benemann J. (1996). Hydrogen biotechnology: Progress and prospects. *Nature Biotechnology* **14**(9):1101-1103.

Billings, R.E. (1991). The hydrogen world view. Washington, DC: *American Academy of Science*. **pp** 66–76.

Bilitewski; B., Härdtle; G. and Marek, K. (1997). Waste Management. Springer, Berlin, ISBN: 3-540-59210-5

Biogas for Better Life, an African Initiative (BBL). (2008). South Africa: Household Biogas Feasibility Study.

Bockris, J.O.M. (1981). The economics of hydrogen as a fuel. *International Journal of Hydrogen Energy*. **6:** 223 (41).

Bockris, J.O.M. (2002). The origin of ideas on a hydrogen economy and its solution to the decay of the environment. *International Journal of Hydrogen Energy*. **27:**731–40.

Calli, B., Mertoglu, B., Inanc, B. and Yenigun, O. (2005) Methanogenic diversity in anaerobic bioreactors under extremely high ammonia levels. *Enzyme Microbiology Technology*. **37:** 448-455.

Cazenave and Neren, (2004) Present-day sea level change: Observations and causes. *Reviews of Geophysics*. **42(3)**: doi: 10.1029/2003RG000139.

Centers for Disease Control and Prevention (CDP) 2010 Climate and Health Program.

Center for Politiske Studier (CEPOS) (2009) Wind Energy. The case of Denmark. Landgreven Copenhagen, Denmark.

Chandrakant, P. and Bisaria, V.S. (1998). Simultaneous bioconversion of cellulose and hemicelluloses to ethanol. *Critical Review of Biotechnology* **18** (4), 295–331.

Chang, J.S., Kuo-Shing Lee, K.S. and Lin, P.J. (2002). Biohydrogen production with fixed bed bioreactors. *International Journal of Hydrogen Energy* **27:** 1167–1174.

Chang, J.J., Chou, C.H., Ho, C.Y., Chen, W.E., Lay, J.J., Huang, C.C. (2008). Syntrophic co-culture of aerobic Bacillus and anaerobic Clostridium for bio-fuels and bio-hydrogen production. *International Journal of Hydrogen Energy*. doi:10.1016/j.ijhydene.

Chen, X., Sun, Y., Xiu, Z., Li, X. and Zhang, D. (2006). Stoichiometric analysis of biological hydrogen production by fermentative bacteria. *International Journal of Hydrogen Energy*. **31:**539-49.

Chong, M.L., Sabaratnam, V.,Shirai,Y. and Hassan, M.A. (2009). Biohydrogen production from biomass and industrial wastes by dark fermentation. Review. *International Journal of Hydrogen Energy*. **34:** 3277-3287.

Clarridge, J.E. (2004). Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases. *Clinical Microbiology Revues*. **17:** 840–862.

Climate Change Information Resources (CCIR). (2005). What causes global climate change?

Clean Fuels Development Coalition (CFDC). (2007). the ethanol fact book.

Climate Institute (CI). Climate Change and Food Security. Summer (2008) Vol 18 N°3.

Czaczyk, K. and Myszka, K. (2007). Biosynthesis of Extracellular Polymeric Substances (EPS) and Its Role in Microbial Biofilm Formation. *Polish Journal. of Environmental Study.* **16 (6):**799-806.

Da Rosa AV. (2005). Fundamentals of Renewable Energy Process. Elsevier Academic Press
Das, D. and Verziroglu, T. N. (2001). Hydrogen production by biological processes: A survey of literature. *International Journal of Hydrogen Energy*. **26:**13-28.

Das, D. (2007). Microbial hydrogen production with Bacillus coagulams IIT-BT S1 isolated from anaerobic sewage sludge, Kotay S M. *Bioresource Technology*. **98(6):** 1183-90.

Das, D., Khanna, N. and Veziroglu, T.N. (2008). Recent developments in biological hydrogen production processes. Chemical Industry and Chemical Engineering Quarterly **14:** (2) 57–67.

Das, D. and Veziroglu, T.N. (2008). Advances in biological hydrogen production processes. *International Journal of Hydrogen Energy***. 33:**6046–57.

Das, D. (2009). Advances in biohydrogen production processes: An approach to commercialization. *International Journal of Hydrogen Energy*. DOI:10.1016/j.ijhydene.2008.12.013.

Da Silva, W.P., Silva, J.A., Macedo, M.R., Macedo, M.R., de Araujo, M.R., Mata, M.M and Gandra, E.A. (2003). Identification of *staphylococcus aureus*, *s. Intermedius* and *s. Hyicus* by pcr Amplification of *coa* and *nuc* genes. *Brazilian Journal of Microbiology*. **34:** 125-127.

Datar, R., Huang, J., Maness, P.C., Mohagheghin, A., Czernik, S. and Chornet, E. (2007). Hydrogen production from the fermentation of corn stover biomass pretreated with a steam-explosion process. *International Journal of Hydrogen Energy*. **32:**932–9.

Davila-Vazquez, G., Arriaga, S., Alatriste-Mondragón, F., de León-Rodríguez, A., Rosales-Colunga, L.M. and Razo-Flores, E. (2008). Fermentative biohydrogen production: trends and perspectives. *Review Environmental Science Biotechnology*. **7:**27-45.

Davis, F. and Higson, S.P.J. (2007). Biofuel cells recent advances and applications. *Biosensors and Bioelectronics* **22:** 1224–1235.

de Vrije, T.d., Haas, G.G.d., Tan, G.B., Keijsers, E.R.P. and Claassen, P.A.M. (2002.). Pretreatment of Miscanthus for Hydrogen Production by Thermotoga elfii. *International Journal of Hydrogen Energy* **27:** (11-12), 1381-1390.

de Vrije, T., Mars. A. E., Budde, M.A, Lai, M.H., Dijkema, C. and Claasen, P.A.M (2007) Glycolytic pathway and hydrogen yield studies of the extreme thermophile *Caldicellulosiruptor saccharolyticus*. *Applied Microbiology Biotechnology*. **74:** 1358 – 1367.

Ding, L., Zhou, Q., Wang, L. and Zhang, Q. (2011). Dynamics of bacterial community structure in a full-scale wastewater treatment plant with anoxic-oxic configuration using 16S rDNA PCR-DGGE fingerprints. *African Journal of Biotechnology*. **10 (4):** 589-600.

Dolfing J. (1987). Microbiological aspects of granular methanogenic sludge. Ph. D. Thesis, Agricultural University Wageningen, The Netherlands, 1987.

Endo, G., Noike, T. and Matsumoto, J. (1982). Characteristics of cellulose and glucose decomposition in acidogenic phase of anaerobic digestion. *Proceeding Society of Civil Engineer*. **325:** 61–68.

Environmental Science Activities for 21st century (ESA21). (2003). Alternative Energy: Solar Energy.

Ercolini, L.A. (2004). PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiology Methods*. **56:**297-314.

Eun, J.S., Fellner, V. and Gumpertz, M.L. (2004). Methane production by mixed ruminal cultures incubated in dual-flow fermentors. *Journal of Dairy Science*. **87:**112-21.

Evans, G. (2001). *Biowaste and biological waste treatment*. The Cromwell press. ISBN: 1-902916-08-5.

Fabiano, B., and Perego, P., (2002). Mathematical modelling and optimization of hydrogen continuous production in fixed bed bioreactor. *Chemical Engineering Science* **57:**3819-3830.

Fang, H.H.P. and Liu, H. (2002). Effect of pH on hydrogen production from glucose by a mixed culture. *Bioresources Technolnology*. **82(1):** 87–93.

Fang, H.H., Zhang, T. and Liu, H. (2002). Microbial diversity of a mesophilic hydrogen-producing sludge. *Applied Microbiology Biotechnology*. **58(1):**112–8.

Fang, H.H.P., Chenlin, L. and Zhang, T. (2006). Acidophilic biohydrogen production from rice slurry. *International Journal of Hydrogen Energy*. **31:**683-92.

Gafan, G.P. and Spratt, D.A. **(**2005)**.** Denaturing gradient gel electrophoresis gel expansion (DGGEGE) an attempt to resolve the limitations of co-migration in the DGGE of complex polymicrobial communities. *FEMS microbiology letters*. **253(2**):303-7.

Gavrilescu M. (2002). Engineering concerns and new developments in anaerobic wastewater treatment. *Clean Technologies and environmental Policy* **3:**346-362.

Gerpen, J. V. (2005). Biodiesel processing and production. *Fuel Processing Technology* **86:** 1097–1107.

Ginkel, S.V., Sung, S. and Lay, J.J. (2001). Biohydrogen production as a function of pH and substrate concentration. *Environmental Science Technology*. **35:**4726-30.

Hallenbeck, P.C. (2009). Fermentative hydrogen production: principles, progress, and prognosis. *International Journal of Hydrogen Energy*. **34:** 7379 – 7389.

Hallenbeck, P.C. and Ghosh, D. (2009). Advances in fermentative biohydrogen production: the way forward? *Trends in Biotechnology*. **5:** 297-297.

Han, S. K. and Shin, H. S. (2004). Biohydrogen production by anaerobic fermentation of food waste. *International Journal of Hydrogen Energy* **29:**569-577.

Handschur, M., Pinar , G., Gallist, B., Lubitz, W and Haslberger, A.G. (2005). Culture free DGGE and cloning based monitoring of changes in bacterial communities of salad due to processing. *Food and Chemical Toxicology*. **43:**1595-1605.

Hansel, A. and Lindblad, P. (1998). Towards optimization of cyanobacteria as biotechnologically relevant producers of molecular hydrogen, a clean and renewable energy source. *Applied Microbiology Biotechnology*. **50:**153–60.

Hasyim, R., Imai, T., Reungsang, A. and O-Thong, S. (2010). Extreme-thermophilic biohydrogen production by an anaerobic heat treated digested sewage sludge culture. *International Journal of Hydrogen Energy*. doi:10.1016/j.ijhydene.2010.06.079.

Haug, R.T. (1993). The practical handbook of compost engineering. Tokyo: Lewis Publishers. **p** 287-334.

Hawkes, F.R., Dinsdale, R., Hawkes, D.L and Hussy, I. (2002). Sustainable fermentative hydrogen production: challenges for process optimization. *International Journal of Hydrogen Energy*. **27:**1339-47.

Hawkes, F.R., Hussy, I., Kyazza, G., Dinsdale, R. and Hawkes, D.L. (2007). Continuous dark fermentative hydrogen production by mesophilic microflora: principles and progress. . *International Journal of Hydrogen Energy*. **32:** 172 – 184.

Herbert, H. P., Fang, H. L. and Zhang, T. (2001). Characterization of hydrogen-producing granules sludge.

Hulshoff Pol, L.W., de Castro Lopes, S.I., Lettinga, G. and Lens, P.N.L. (2004). Anaerobic sludge granulation. *Water Research* **38:** 1376–1389.

Hung, C.H., Lee, K.S., Cheng, L.H., Huang, Y.H., Lin, P.J and Chang, J.S. (2007). Quantitative analysis of a high-rate hydrogen-producing microbial community in anaerobic agitated granular sludgebed bioreactors using glucose as substrate. *Applied Microbiology Biotechnology*. **75:**693-701.

Idaho National Laboratory (INL). (2011). Hydropower Resource Assessment at existing Reclamation Facility.

International Energy Agency (IEA). (2005). Renewable Energy: RD and D Priorities. Insights from IEA Technology Programmes.

International Energy Agency (IEA). (2007a). Biofuel production. *Energy Technology Essentials*.

International Energy Agency (IEA). (2007b). Renewable for Heating and cooling. *Renewable Energy Technology Deployment.*

International Energy Agency (IEA). (2009). Bioenergy- a sustainable and Reliable Energy Source. Main Report.

Intergovernmental Panel on Climate Change (IPCC) (2007) Climate Change 2007: The physical Science Basis, Summary for Policymakers. Contribution of Working Group 1 to the

fourth Assessment Report of the intergovernmental Panel on Climate Chamge, 21 pp, Geneva. Download from http://www.ipcc.ch on Februery 21, 2007.

Iyer, P., Ann Bruns, M., Zhang, H., Van Ginkel, S. and Logan, B.E. (2004). Hydrogen Producing bacterial communities from a heat-treated soil inoculum. *Applied Microbiology Biotechnology*. **66:**166-173.

Kadar, Z., de Vrijek, T., van Noorden, G.E, Budde, M.A.W., Szengyel, Z. and Reczey, K. (2004). Yields from glucose, xylose, and paper sludge hydrolysate during hydrogen production by the extreme thermophile Caldicellulosiruptor saccharolyticus. *Applied Biochemistry Biotechnology*.**113:**497–508

Knapp, L.A. (2005). Review article: Denaturing gradient gel electrophoresis and its use in the detection of the major histocompatibility complex polymorphism. *Tissue Antigens*. **65:**211-219.

Kapdan, I.K. and Kargi, F. (2006). Bio-hydrogen production from waste materials. *Enzyme Microbiology Technology.* **38:** 569–582.

Karadag, D. and Puhakka, J.A. (2010). Effect of changing temperature on anaerobic hydrogen production and microbial community composition in an open-mixed culture bioreactor. *International Journal of Hydrogen Energy*. **35 (20):** 10954–10959.

Karube, I., Urano, N., Matsunaga, T. and Suzuki, S. (1982) Hydrogen production from glucose by immobilized growing cells of *Clostridium butyricum*. *European Journal Applied Microbiolology*. **16:**5–9.

Kotsopoulos, T., Zeng, R. J. and Angelidaki, I. (2006). Biohydrogen Production in Granular Up-Flow Anaerobic Sludge Blanket (UASB) Reactors with Mixed Cultures under Hyper-Thermophilic Temperature (70°C). *Biotechnology and Bioengineering*. DOI 10.1002/bit.

Kraemer, J.T. and Bagley, D.M. (2007). Improving the yield from fermentative hydrogen production. *Biotechnology Letter*. **20:** 685-95.

Kerr, P.S., Huber, S.C. and Israel, D.W. (1984). Effect of N-source on soybean leaf sucrose phosphate synthase, starch formation, and whole plant growth. *Plant. Physiology*.**75:** 483–488.

Kuroda, K., Silveira, R. G., Nishio, N., Sunahara, H. and Nagai, S. (1991). Measurement of dissolved hydrogen in an anaerobic digestion process by a membrane-covered electrode. *Journal of Fermentation and Engineering*. **71:** 418 – 421.

Kyazze, G., Martinez-Perez, N., Dinsdale, R., Premier, G.C., Hawkes, F.R., Guwy, A.J. and Hawkes, D.L. (2005). Influence of Substrate Concentration on the Stability and Yield of Continuous Biohydrogen Production. *Wiley InterScience*. DOI: 10.1002/bit.20802.

LaPara, T.M., Konopka, A., Nakatsu, C. and Alleman, J.E. (2000). Effects of elevated temperature on bacterial community structure and function in bioreactors treating a synthetic wastewater. *Journal of Industrial Microbiology and Biotechnology* **24**: 140-145.

Lay, C.H., and Lin, C.Y. (2004). Carbon/nitrogen-ratio effect on fermentative hydrogen production by mixed microflora. *International Journal of Hydrogen Energy*. **29:**41-45.

Lawson, Anani Soh, A., Ralambotiana, H., Ollivier, B., Prensier, G., Tine, E. and Garcia. J.L. (1991). *Clostridìum thermopalmarìum* sp. a Moderately Thermophilic Butyrate-Producing Bacterium Isolated from Palm Wine in Senegal System. *Applied Microbiolology*. **14:**135-139.

Lee, M.J. and Zinder, S.T. (1988). Hydrogen partial pressures in thermophilic acetate- 426 oxidizing methanogenic coculture. *Applied Environmental Microbiology*. **54:** 1457–1461.

Lee, K-S., Lo, Y.S., Lo, Y.C., Lin, P.J. and Chang, J.S. (2003). Hydrogen production with anaerobic sludge using activated-carbon supported packed-bed bioreactors. *Biotechnology Letter*. **25:**133–138.

Lee, K.S., Wu, J.F., Lo, Y.S., Lo, Y.C., Lin, P.J. and Chang, J.S. (2004). Anaerobic biohydrogen production with an efficient carrier-induced granular sludge bed bioreactor. *Biotechnology and Bioengineering* **87:** 648 – 657.

Lee, K.S., Lin, P.J and Chang, J.S. (2005). Temperature effects on biohydrogen production in a granular sludge bed induced by activated carbon carriers. *International Journal of Hydrogen Energy*.

Lee, K.S., Lo, Y.C., Lin, P.J. and Chang, J.S. (2006). Improving biohydrogen production in a carrier-induced granular sludge bed by altering physical configuration and agitation pattern of the bioreactor. *International Journal of Hydrogen Energy* .**31:** 1648 – 1657.

Lee, Y.J., Romanek, C.S. and Wiegel, J. (2007). Clostridium aciditolerans sp. and acid-tolerant spore-forming anaerobic bacterium from constructed wetland sediment. *International Journal of Systematic and Evolutionary Microbiology*. **57:**311-5.

Levin, D. B.; Pitt, L. and Love, M. (2004). Biohydrogen production: Prospects and limitations to practical application. *International Journal of Hydrogen Energy* **.29:** (2) 173–185.

Levin, D.B., Islam, R., Cicek, N., and Sparling, R., (2006) Hydrogen production by Clostridium thermocellum 27405 from cellulosic biomass substrates. *International Journal of Hydrogen Energy* **31**:1496–503.

Li, C. L. and Fang, H. H. P. (2007). Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. *Critic Revue of. Environmental. Science. Technology*.**37:** (1), 1–39.

Li, R.Y., Zhang, T. and Fang, H.H.P. (2011). Application of molecular techniques on heterotrophic hydrogen production research. *Bioresource Technology*. **102:** 8445–8456.

Lin, Y., Wu, C.C. and Hung, C.H. (2008). Temperature effects on fermentative hydrogen production from xylose using mixed anaerobic cultures. *International Journal of Hydrogen Energy*. **33:**43-50.

Liu, Y., Xu, H., Yang, S. and Tay, J. (2003). Mechanisms and models for anaerobic granulation in upflow anaerobic sludge blanket reactor. *Water Research*. 37:661-673.

Liu, Y. and Tay, J.H. (2004). State of the art of biogranulation technology for wastewater treatment. *Biotechnology Advances*. **22:**533-563.

Liu, X., Ren, N., Song, F., Yang, C. and Wang, A. (2008). Recent advances in fermentative biohydrogen production. *Progress in natural science*. **18:** 253 – 258.

Lovely, D.R. (2006). Microbial fuel cells: novel microbial physiologies and engineering approaches. *Current Opinion in Biotechnology* **17** (3)**:** 327–32.

Lynd, L.R., Grethlein, H.G. and Wolkin, R.H. (1989). Fermentation of cellulose substrates in batch and continuous culture by Clostridium thermocellum. *Applied Environmental Microbiology*. **55:**3131–9.

Lynd, L.R., Weimer, P.J., Zyl, W.H. and Pretorius, I.S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. *Microbioliology Molecular Biology Review*. **66:**506–77.

Mata-Alvarez, J. (2003). Biomethanization of the organic fraction of municipal solid wastes.

McLamb, E. (2011). Fossils Fuels Vs Renewable Energy Resources. *Ecology Global Network*. Today.

Miller, N., Bryant, J.E and Madsen, E.L. (1999). Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Applied Environmental Microbiology*. **65:**47 15-4724.

Moore, R.B and Raman V. (1998). Hydrogen infrastructure for fuel cell transportation. *International Journal of Hydrogen Energy*. **23**:617–20.

Muyzer, G., De Waal, E.C. and Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturating gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S rRNA. *Applied Environmental Microbiology*. **59:**695–700.

Muyzer, G. and Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology impact factor*. **73:**127–141.

Nath, K. And Das, D. (2011). Modeling and optimization of fermentative hydrogen production. *Bioresources Technology*. in press.

Ngoma, L., Masilela, P., Obazu, F. and Gray, V.M. (2011). The effect of temperature and effluent recycle rate on hydrogen production by undefined bacterial granules. *Bioresource Technology*. **102:** 8986–8991.

Ni, M., Leung, D.Y.C., Leung, M.K.H. and Sumathy, K. (2006). An overview of hydrogen production from biomass. *Fuel Processing Technology* **87:** 461–472.

Niu, K., Zhang, X., Tan, W.S. and Zhu, M.L. (2011). Effect of culture conditions on producing and uptake hydrogen flux of biohydrogen fermentation by metabolic flux analysis method. *Bioresources Technololgy*. In press.

Ostrem, K. (2004): Greening Waste: Anaerobic Digestion For Treating The Organic.

O-Thong, S., Prasertsan, P., Karakashev, D. and Angelidaki, I. (2008). High-rate continuous hydrogen production by *Thermoanaerobacterium thermosaccharolyticum* PSU-2 immobilized on heat-pretreated methanogenic granules. *International Journal of Hydrogen Energy* **33:** 6498–6508.

Pauss, A., Samson, R., Guiot, S.R. and Beauchemin. C. (1990). Continuous measurement of dissolved H_2 in anaerobic digestion using a new hydrogen/air fuel cell probe. *Biotechnology. Bioengineering*. **35:**492-501.

Perrings, C. (2010). Biodiversity, Ecosystem Services, and Climate Change. *The Economic Problem*. Environmental Economics Series. 120.

Planethopia (2011). Fossil fuels depletion in future.

Rahmstorf, S., Cazenave, A., Church, J.A., Hansen, J.E., Keeling, R.F., Parker, D.E. and

Somerville, R.C.J. (2007). Recent Climate Observations Compared to Projections. Science Vol 316 May 2007p709.

Reith, J.H., Wijffels, R.H and Barten, H. (2003). Bio-methane and biohydrogen. Status and perspective of biological methane and hydrogen production, Dutch biological hydrogen foundation, Wageningen.

Renewable Energy Policy Network for 21st Century (REN 21). (2011). Global Status Report.

Rittmann, B.E. (2008). Opportunities for renewable bioenergy using microorganisms. *Biotechnology and Bioenergy*. **100:**203 – 212.

Robe, P., Nalin, R., Capellano, C., Vogel, T.M and Simonet, P. (2003). Extraction of DNA from soil. European Journal of Soil Biology **39:**183–190.

Rutz, D., and Janssen, R. (2007). Biofuel Technology Handbook. *WIP Renewable Energy*, Germany.

Sahlstrom, L. (2003). A review of survival of pathogenic bacteria in organic waste used in biogas plants. *Bioresource Technology* **87(2):**161-166.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology Evolution*. **4:**406–425.

Schneegurt, M.A., Dore, S.Y. and Kulpa, C.F. (2003). Direct Extraction of DNA from Soils for Studies in Microbial Ecology. *Current Issues in Molecular Biology*. **5:**1-8.

Sekiguchi, H., Tomioka, N., Nakahara, T.and Uchiyama, H. (2001). A single band does not always represent single bacterial strains in denaturing gradient gel electrophoresis analysis. *Biotechnology Letter*. **23:**1205-1208.

Setlow, P. (2003). Spore germination. *Current Opinion Microbiology*. **6:**550-6.

Smith, J.M., Van Ness, H.C. and Abbott, M.M. (2000). Introduction to chemical engineering thermodynamics. McGraw-Hill, New York.

Solomon, B.O., Zeng, A.P., Biebl, H., Schlieker, H., Posten, C. and Deckwer, W.D. (1995). Comparation of the energetic efficiencies of hydrogen and oxychemicals formation in *Klebsiella pneumoniae* and *Clostridium butyricum* during anaerobic growth on glycerol. *Journal of Biotechnology*. **39:**107-17.

Sommer, P., Georgieva, T. and Ahring, B.K. (2003) Potential for using thermophilic anaerobic bacteria for bioethanol production from hemicellulose. *Biochemical Society Transactions***: 32:**283

Stackbrandt, E and Goebel, B.M. (1994). Taxonomic note: a place for DNA- DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal System Bacteriology*. **44:**846–849.

Stronach, S.M., Rudd, T and Lester, J.N. (1986). Anaerobic digestion processes in industrial wastewater treatment. *Biotechnology Monographs*. Springer-Verlag, Berlin.

Suzuki, Y. (1982). On hydrogen as fuel gas. *International Journal of Hydrogen Energy* **7:**227.

Svensson, B. and Karlsson, A. (2005). Dark fermentation for hydrogen production from organic wastes. In: Lens P, Westermann.

Taguchi, F., Mizukami, N., Saito-Taki, T. and Hasegawa, K. (1995). Hydrogen production from continuousfermentation of xylose during growth of Clostridium sp. strain No. 2. *Canada Journal of Microbiology*. **41:**536–40.

Tanisho, S. and Kamiya, N. (1989). Hydrogen evolution of Enterobacter aerogenes depending on culture pH: mechanism of hydrogen evolution from NADH by means of membrane-bound hydrogenase. Biochimical Biophysic Acta. **973:** 1-6.

Theron, A. K. and Rossouw, M. (2008). Analysis of potential coastal zone climate change impacts and possible response options in the southern African region. Science Real and Relevant: 2nd CSIR Biennial Conference, CSIR International Convention Centre Pretoria, 17-18 November 2008, 10pp.

Thompson, L.J. (2005). Biological hydrogen production using an anaerobic fluidized bed bioreactor. Master thesis. University of the Witwatersrand, Johannesburg.

Thompson, L.J., Gray, V.M., Kalala, B., Lindsay, D and Reynolds, K. (2008). Biohydrogen production by Enterobacter cloacae and Citrobacter freundii in carrier induced granules. *Biotechnology Letter*. 30: 271-274.

Tsai, W.T., Lin, C.C.and Yeh, C.W. (2007). An analysis of Biodiesel fuel from Waste Edible oil in Taiwan. *Renewable Sustainable Energy Review*. **11:** (5) 838-857.

Tay, J.H., Tay, S.T.L., Liu, Y., Show, K.Y. and Ivanov, V. (2006). Biogranulation technologies for wastewater treatment. ISBN: 9780080450223. **6:**1-277.

Tysganov, A.A. (2007). Biological generation of hydrogen. *Russian Journal of General Chemistry* .**77:** 685-693.

Ueno, Y., Haruta, S., Ishii, M. and Igarashi, Y. (2001a). Characterization of a microorganism isolated from the effluent of hydrogen fermentation by microflora. *Journal Bioscience Bioengineering*. **92:**397–400.

Ueno, Y., Haruta, S., Ishii, M. and Igarashi, Y. (2001b). Microbial community in anaerobic hydrogen-producing microflora enriched from sludge compost. *Applied Microbiology Biotechnology*. **57:**555-62.

United National Environment Programmes (UNEP) (2010). Integrated Solutions for Biodiversity, Climate Change and Poverty.

Urbaniec, K. and Grabarczyk, R. (2009). Raw materials for fermentative hydrogen production. *Journal of Cleaner production* **17:** 959-962.

US Department of Energy (US DOE). (2010). Energy Efficiency and Renewable Energy.

US Energy Information Administration (US EIA). (2009). Fossil Fuels.

US Environmental Protection Agency (US EPA). (2011). Agriculture and Food Supply.

Valdez-Vazquez, I., Rios-Leal, E., Esparza-Garcia, F., Cecchi, F. and Poggi-Varaldo, H.M. (2005). Hydrogen production from the organic fraction of municipal solid waste using semi-continuous solid substrate anaerobic reactors: influence of the temperature. *International Journal of Hydrogen Energy*. **30:**1383–1391.

Valdez-Vazquez, I. and Poggi-Varaldo, H.M. (2009) Hydrogen production by fermentative consortia. *Renewable and Sustainable Energy Reviews* **13:** 1000–1013.

Valentine, D.L., Reeburgh, W.S. and Blanton, D.C. (2000). A culture apparatus for maintaining H2 at sub-nanomolar concentrations. *Journal of Microbiological Methods*.**39:** 243 – 251.

Van Groenestijn, J.W., Geelhoed, J.S., Goorissen, H.P., Meesters, A.J.M., Stam, A.J.M. and Claassen, P.A.M. (2009). Performance and population analysis of a non-sterile trickle bed reactor inoculated with *Caldicellulosiruptor saccharolyticus*, a thermophilic hydrogen producer. *Biotechnology and Bioengineering*.**102:** 1361 – 1367.

Van Niel, E.W.J., Budde, M.A.W., De Haas, G.G., Van der Wal, F.J., Claasen, P.A.M. and Stams, A.J.M. (2002). Distinctive properties of high hydrogen producing extreme thermophiles, Caldicellulosiruptor saccharolyticus and Thermotoga elfii. *International Journal of Hydrogen Energy* **27(11-12):**1391-1398.

Van Niel, E.W.J., Budde, M.A.W., de Haas, G.G., van de Wal, F.J., Claassen, P.A.M., Stams,

Van Ootegehem S.A., Jones A, van der Lelie D. and Dong B, Mahajan D. (2004). Hydrogen production and carbon utilization by *Thermotoga neapolitana* under anaerobic and microanaerobic growth conditions. *Biotechnology Letters* **26:**1223-1232.

Valentine, D.L., Reeburgh, W.S. and Blanton, D.C. (2000). A culture apparatus for maintaining H2 at sub-nanomolar concentrations. *Journal of Microbiology Method*. **39:** 243–251.

Veziroglu, T.N. and Barbir, F. (1995). Transportation Fuels-Hydrogen Energy Technology and the environment, vol 4, *Wiley-Interscience* 2712-2730.

Vindis, P., Mursec, B., Janzekovic, M. and Cus, F. (2009). The impact of mesophilic and thermophilic anaerobic digestion on biogas production. *Journal of achievements in materials and manufacturing Engineering*. **36:**2.

Wang, J. and Wan, W. (2008a). Effect of Fe^{2+} concentrations on fermentative hydrogen production by mixed cultures. *International Journal of Hydrogen Energy* **33:**1215.

Wang, J. and Wan, W. (2008b). Effect of temperature on fermentative hydrogen production by mixed cultures. *International Journal of hydrogen Energy*. **33:**5392-7.

Wang, J. and Wan, W. (2009).Factors influencing fermentative hydrogen production: A review. *International journal of hydrogen energy*. **34:** 799-811.

Warrick, R.A., Bhuiya, A.H. and Mirza, M.Q. (1993). Climate Change and Sea-level Rise: the Case of the Coast. Briefing Document No. 6, Bangladesh Unnayan Parishad (BUP), Dhaka.

Weimer, P.J. and Zeikus, J.G. (1977). Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence and presence of *Methanobacterium thermoautotrophicum*. *Applied Environmental Microbiology*. **33:**289-297.

Wiegel, L., Ljuizgduhl, L. G. and Rawson, J. R. (1979). Isolation from soil and properties of the extreme thermophile *Clostridium thermohydrosulfuricum.* Journal of Bacteriology. **139:** 800-810.

Wilkie, A.C., Riedesel, K.J. and Owens, J.M. (2000). Stillage Characterization and Anaerobic Treatment of Ethanol stillage from Conventional and cellulosic feedstocks. *Biomass Bioengineering* **19:** 63-102.

Woodward, J., Mattingly, S.M., Danson, M., Hough, D., Ward, N. and Adams, M. (1996). In vitro hydrogen production by glucose dehydrogenase and hydrogenase. *Nature Biotechnology*. **14** (7)**:** 872–874.

World Bank Group (WBG). (2009). Direction in hydropower.

World Health Organization (WHO). (2010). Climate change and health. World Health Report, Geneva.

Wu, S.Y., Hung, CH., Lin, C.N., Chen, H.W., Lee, A.S., and Chang, J.S., (2006) Fermentative hydrogen production and bacterial community structure in high-rate anaerobic bioreactors containing silicone-immobilised and self-flocculated sludge. *Biotechnol Bioengineering*. **93**:934–46.

Xing, D.F., Ren, N.Q. and Rittmann, B.E. (2008). Genetic diversity of hydrogen-producing bacteria in an acidophilic ethanol–H_2-coproducing system, analyzed using the [Fe]-hydrogenase gene. *Applied Environnemetal Microbiology* **74:**1232–9.

Yokoi, H., Ohkawara, T., Hirose, J., Hayashi, S. and Takasaki, Y. (1995). Characteristics of hydrogen production by aciduric *Enterobacter aerogenes* Strain HO-39. *Journal of fermentation and Bioengineering*. **80:**571-574.

Yokoi, H., Maki, R., Hirose, J., and Hayashi, S., (2002) Microbial production of H2 from starch-manufacturing wastes. *Biomass Bioenergey*. **22**:389–95.

Zeiden, A.A. and van Niel, E.W.J. (2010). A quantitative analysis of hydrogen production efficiency of the extreme thermophile *Caldicellulosiruptor owensis* OLT. *International Journal of Hydrogen Energy*. **35:** 1128–1137.

Zeikus, J.G. (1980). Chemical and fuel production by anaerobic bacteria. *Annual Review Microbiology*. **34:** 423-464.

Zinder, S.H. and Koch, M. (1984). Non-aceticlastic methanogenesis from acetate:acetate oxidation by a thermophilic syntrophic coculture. *Archive of Microbiology*. **138:** 263–272.

Zhang, T., Liu, H. and Fang, H.H.P. (2003). Biohydrogen production from starch in wastewater under thermophilic conditions. *Journal Environmental Management* .**69:**149–56.

Zhang, Z.P., Show, K.Y., Tay, J.H., Liang, D.T., Lee, D.J and Jiang, W.J. (2007a). Rapid formation of hydrogen-producing granules in an anaerobic continuous stirred tank reactor induced by acid incubation. *Biotechnology* **96:** 1040 – 1050.

Zhang, Z.P., Tay, J.H., Show, K.Y., Yan, R., Liang, D.T., Lee, D.J. and Jiang, W.J. (2007b). Biohydrogen production in a granular activated carbon anaerobic fluidized bed reactor. *International Journal of Hydrogen Energy* **32:** 185 – 191.

Zhang, Z.P., Adav, S.S., Show, K.T., Tay, J.H., Liang, D.T., Lee, D.J. and Su, A. (2008a). Characterization of rapidly formed hydrogen-producing granules and biofilm. *Biotechnology and Bioengineering.*

Zhang, Z.P., Show, K.Y., Tay, J.H., Liang, D.T. and Lee, D.J. (2008b). Biohydrogen production with anaerobic fluidized bed reactors–A comparison of biofilm-based and granule-based systems. *International Journal of Hydrogen Energy*. **33:** 1559 – 1564.

Zhang, Z.P., Show, K.Y., Tay, J.H., Liang, D.T.and Lee, D.J. (2008c). Enhanced continuous biohydrogen production by immobilized anaerobic microflora. *Energy Fuels* **22:** 87 – 92.

Appendices

Appendix I:

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
NH4HCO	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 x 10

Gel electrophoresis Agarose Gel (50 ml)

Agarose (0.5g for 1%)

5X TBE: 10ml

Distilled water: 40ml

Heat until agarose has completely dissolved

Add 1 µl Ethidium Bromide

5X TBE

Tris base: 54g

Boric acid: 27.5g

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

5X TBE: 100ml

Sterile distilled water: 900ml

Ethidium Bromide: 2.5 µl

10% Ammonium persulfate (APS)

APS: 100mg

H_2O: 1ml

Store at -20ºC

TE Buffer

Tris-HCl: 10 mM, pH 8.0

EDTA: 1 mM

Sterilize solutions by autoclaving for 20 min at 15 psi (1.05 kg/cm2) on liquid cycle.

Store the buffer at room temperature.

Appendix II: Provisional patent application receipt

PATENTS ACT, 1978

APPLICATION FOR A PATENT AND ACKNOWLEDGEMENT OF RECEIPT

[Section 30(1) – Regulation 22]

FILED

Revenue stamps or franking machine impression

ting of a patent is hereby requested by the undermentioned applicant, ion filed in duplicate

Application No.: 01 2010/04063

Applicant's or agent's reference: CAVENY

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

Address(es) of applicant(s): 1 Jan Smuts Avenue, Johannesburg

Title of invention: Effluent Gas-discharger

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

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

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

This application is accompanied by:

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.
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
10. A declaration and power of attorney on Form P9.
11. Request for antedating on Form P4.
12. Request for classification on Form P9.

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

this 7th day of June 2010

Signature of applicant(s) or agent

Received REGISTRAR OF PATENTS, DESIGNS, TRADE MARKS AND COPYRIGHT

Registrar of Patents 07 JUN 2010