DEVELOPMENT AND TESTING OF A BIOREACTOR FOR PRODUCTION OF HYDROGEN

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A dissertation submitted to the Faculty of Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science

Johannesburg 2007

DECLARATION

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

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ABSTRACT

A laboratory-scale anaerobic Fluidised Bed Bioreactor (FBBR) was designed and constructed for hydrogen gas (H_2) production using a sucrose-based synthetic wastewater. In the first experiment, the anaerobic FBBR was inoculated with two facultative anaerobic bacteria *Citrobacter freundii* (Cf1) (Accession number: EU046372) and *Enterobacter cloacae* (Ecl) (Accession number: EU046373) to study their H_2 productivity capacity. Granulated activated carbon was used to initiate the growth and development of bacterial granules. For granule production the hydraulic retention time (HRT) was gradually reduced from 8 to 0.5 h. Hydrogen production and sucrose consumption was investigated at HRTs ranging from 8 to 0.5 h. Sucrose was converted into volatile fatty acids (VFAs) and biogas (essentially H_2). Temperature and pH of the anaerobic FBBR were controlled at 37±1ºC and 5.6±0.1 respectively. The H_2 production rate (HPR) reached 138mmol/(h.L) at 0.5 h HRT. Acetic, butyric and propionic acids were detected at 104.5±21.06, 76.13±16.81 and 24.91±2.67 mg/L respectively. Results showed that Ecl and Cf1 were able to convert sucrose into soluble and biogas products with high rate of H_2 gas production.

In the second experiment, a heat and acid treated sample of activated sewage sludge from an anaerobic sewage works was used as the inoculum for growing the granular bed in the anaerobic FBBR. The anaerobic FBBR was operated according to conditions described in the first experiment. HPR reached a maximum of 130.1 mmol/(h.L) at 0.5 h HRT with constant influent sucrose concentration of 17.65 g/L. In both experiments the influent sucrose concentration in the bioreactor expressed in terms of chemical oxygen demand (COD) was 20 gCOD/L. Optimal sucrose to hydrogen ration was observed at a HRT of 2 h and led to a H_2 yield (YH_2) of 1.61 mmol-H_2/mmol-sucrose.

AKNOWLEDGMENTS

I would like to thank the following for their help and support without which this work could not been completed.

My supervisors, Prof V. M. Gray, Prof D. Hildebrandt and Prof D. Glasser, for their genius advices, encouraging suggestions and extreme patience manifested during the present project completion.

Dr D. Lindsay, for her prompt and fruitful suggestions when consulted.

Dr. Tommy van der Merwe, Dave Amm and Basil Chassoulas for their technical support on setting up of GC, bioreactor construction and electronic process control (Data logger) respectively.

My family, for their love, faith and endless support that I beneficiated along my entire life.

My colleagues and friends, for their help with the methodology in microbiology and the family climate created in my restricted environment.

Eskom Technology Services International (TSI) Pty (Ltd), for funding which helped for project expenses and survival.

Centre of Materials and Process Synthesis (Comps), for their support for facilities and research environment beneficiated during this project completion.

PUBLICATION

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

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LIST OF ABBREVIATIONS

Units

For all parameters SI units are used, except for time and temperature where they are expressed in hour (h) and ºC respectively.

Abbreviations

ATP	Adenosine triphosphate
CO_2	Carbon dioxide
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
DNA	Dinucleotide Acid
ε	Expansion expressed in percentage
EDTA	Ethylenediamine Tetraacetic
FBBR	Fluidised bed bioreactor
Fd	Ferredoxin, oxidised
FdH	Ferredoxin, reduced
FID	Flame ionisation detector
GAC	Granular activated carbon
GC	Gas chromatograph
H	Average bed height
H_2O	Water
H_o	Static bed height
HPR	Hydrogen production rate
HRT	Hydraulic retention time
NA	Nutrient agar
NAD	Nicotinamide adenine dinucleotide, oxidised
NADH	Nicotinamide adenine dinucleotide, reduced

NB	Nutrient broth
O_2	Oxygen gas
OLR	Organic loading rate
ORP	Oxido-reduction potential
OD	Optical density
PCR	Polymerase chain reaction
Redox	Reduction - oxidation
SEM	Scanning electron micrograph
SHPR	Specific hydrogen production rate
TAE	Tris-Acetate-EDTA
TCD	Thermal conductivity detector
UASB	Upflow anaerobic sludge bed reactor
VFAs	Volatile fatty acids
VSS	Volatile suspended solids
YH_2	Hydrogen yield

CHAPTER ONE – INTRODUCTION

I.1 OBJECTIVES

The main objective of this research was to produce H_2 gas using liquid waste materials in an anaerobic FBBR.

The specific objectives were to:

- design, construct and operate an anaerobic FBBR system based on bacterial granules for the production of biogas in the form of H_2 using sucrose as the carbon substrate.

- initiate the growth and development of a fluidised bed of bacterial granules.

- investigate the performance of the system at different HRTs using an anaerobic FBBR inoculated with two bacterial species suitable for degrading organic materials contained in synthetic liquid waste (H_2O acclimated with sucrose as carbon source).

- evaluate the effectiveness of H_2 production using a mixed undefined culture derived from activated sewage sludge in order to simulate a potential industrial application.

1.2 LITERATURE REVIEW

1.2.1 Renewable Energy

Hydrocarbons from fossil fuels such as oil, coal and natural gas are responsible for up to 80% of world's energy production (Das & Veziroğlu, 2001, Zurawaski *et al*., 2005, Leite *et al*., 2006, Basak & Das, 2007). The combustion of fossil fuels for energy production, electricity generation, transportation, or other industrial processes releases carbon dioxide (CO_2) and other greenhouse gases into the atmosphere, thereby impacting negatively on the environment (Metz *et al*., 2005).

In recent years, global warming and associated climate change have been found to be mainly due to the increase of CO_2 concentration into the atmosphere. This has become a matter of growing concern all over the World. Research has been initiated to reduce or to stabilise the atmospheric CO_2 level and greenhouse gas emission (Reith *et al*., 2003, Metz *et al*., 2005).

In addition to CO_2 emission, it is apparent that the current use of fossil fuels has greatly increased due to a high energy demand resulting from global population growth, global economic expansion and energy-based increased standards of living (Stout *et al.*, 2001). Many fossil fuels reserves are at their peak of extraction and their production is rigorously controlled by a small cartel of very powerful nations who decide on pricing schedules (Chow *et al*., 2003, Zurawaski *et al*., 2005). Furthermore, fossil fuels are recognised as non renewable sources of energy. A simple observation of the rules of economic supply and demand indicates that our dependence on oil-based production is unsustainable (Das & Veziroğlu, 2001, Crabtree *et al*., 2004, Kapdan & Kargi, 2006).

A combination of the situation presented above, the current increase in global oil prices and the subsequent environmental issues have boosted the impetus for research

on alternative energy sources to complement or possibly substitute conventional energies of fossil origin (Das & Veziroğlu, 2001).

Carbon capture and sequestration in geologic formations or in terrestrial ecosystems have been developed to reduce the amount of CO_2 released into the atmosphere (Chow *et al*., 2003, Simbeck, 2004, Azar *et al*., 2006). This process can be combined with the actual H_2 energy production from fossil fuels which has been efficiently developed. The process is still under intensive research. It is expensive for small fossil-fuelled plants and still has safety issues for public use (Metz *et al*., 2005, Riis *et al*., 2005).

Solar, wind and nuclear power; CH_4 and H_2 gases are considered as alternative sources for substituting energy production from fossil fuels (Jacobsson & Johnson, 2000, Chow *et al*. 2003, Elhadidy & Shaahid, 2003). Actually, greater interest has been expressed in the use of H_2 gas because other renewable energy sources have some undesirable effects (e.g. waste disposal for nuclear energy) or their energy production is low for industrial and commercial applications (e.g. solar and wind powers) (Padró & Putsche, 1999). The use of H_2 can herald the inauguration of the new "Hydrogen Economy" generation (Crabtree *et al*., 2004, Bossel *et al*., 2007).

H_2 is seen as energy of the future. There is an abundance of biomass based substrates for H_2 generation on earth. These substrates are distributed throughout the world and without regard for national boundaries. Unlike the case with fossil fuels, H_2 is not a primary source of energy: it has to be extracted from other sources such as water or organic materials (Das & Veziroğlu, 2001, Dunn, 2002, Simbeck, 2004, Lin *et al.*, 2007). The latter would provide an additional benefit with regard to waste disposal. H_2 exhibits higher energy content per unit weight than any other fuel energy source known (122 kJ/g) (Han & Shin, 2004, Zhang & Shen, 2005). Its combustion releases high amounts of energy with H_2O as a by-product (Das & Veziroğlu, 2001). H_2 is considered as a clean and renewable source of energy without any detrimental effect

to the environment. It can be used directly for energy production or it can be converted into electricity by use of fuel cells (Lay, 2001, Basak & Das, 2007).

1.2.2. Hydrogen uses and production

Uses of Hydrogen

H_2 has many applications mostly in industrial sectors. Some of them are presented below:

Chemical feedstocks

H_2 is used as feedstocks in numerous applications such as production of ammonia for nitrogen fertiliser in the Haber process (this process uses 50% of the world's H_2 production), production of hydrogen peroxide, cyclohexane and similar products from aromatic or ring components. (Ramachandran & Menon, 1998, Hemmes *et al*., 2003)

Hydrogenation of oils

A number of industrial and consumer products use H_2 in the hydrogenation process. Some examples of these applications are hydrogenation of unsaturated fatty acids such as vegetable oils, fish oil or palm oil for production of margarine and other food products where they are converted to the solid state from liquid form. In this process high purity of H_2 is required (Ramachandran & Menon, 1998, Hemmes *et al*., 2003, Dutta *et al*., 2005).

Oil refining

H_2 is used for cracking of more viscous oils to lower molecular weight products such as gasoline and diesel and for removing contaminants such as sulphur (Ramachandran & Menon, 1998, Hemmes *et al*., 2003, Ogden, 2004).

High temperature flames

Combustion of pure H_2 is used in high temperature flames (over 2000°C) in specialised fabrication and processing such as glass and quartz cutting, high temperature welding and germs processing (Hemmes *et al*., 2003).

Furnace atmospheres

In the production of high quality products such as semi-conductors in electronic applications, sinters and compacts, glass and ceramics in float processes; H_2 is used as furnace atmosphere and carrier gas to avoid any contamination and to prevent oxidation of the large tin bath. H_2 is also used as a reducing agent in furnaces in uranium oxide and in most oxide reduction processes (Ramachandran & Menon, 1998, Hemmes *et al*., 2003).

H_2 as O_2 scavenger

H_2 is used to purify gases (e.g. argon) that contain trace amounts of O_2, using catalytic combination of the O_2 and H_2 followed by removal of the resulting water (Ramachandran & Menon, 1998, Hemmes *et al*., 2003).

H_2 as a fuel

The choice of H_2 as a fuel was due to its higher energy content and low molecular weight compared to fuel of carbon origin (Reith *et al*., 2003). H_2 in liquid form has been used for a long time as a propellant in the aerospace industry and in rocket engines (Ramachandran & Menon, 1998, Hemmes *et al*., 2003). H_2 as a gas can be used in fuel cells in which it is combined with O_2 from air by a chemical reaction to produce electricity. This process is under current research and development, it is the one which can allow H_2 to be used for transportation in the future environmentally friendly world (Reith *et al*., 2003, Lin *et al.*, 2007).

Hydrogen production

H_2 can be produced by a number of physico-chemical processes, among them chemical and thermochemical processes are used at industrial and commercial scales. Unfortunately, they are recognised as non-environmentally friendly, expensive or energy intensive (Reith *et al.,* 2003).

Biological production of H_2 is seen to be a potential and more attractive way especially if waste materials could be used as raw material (Leite *et al*., 2006). Generation of H_2 from biological materials, especially lignocellulosic materials, has became the focus of current research. This represents a potential route towards the development of sustainable energy production processes (de Vrije *et al*., 2002, Lin *et al.,* 2007).

Chemical and thermochemical H_2 production

Steam reforming of natural gas or hydrocarbon, partial oxidation of hydrocarbon, coal gasification and electrolysis of H_2O are processes used for H_2 production. A short description of these processes is presented below.

Steam reforming. This process involves the conversion of natural gas (CH_4) or hydrocarbon into H_2 and CO_2 in the presence of H_2O vapour. This reaction is carried out in two steps. The first step is a catalytic conversion of hydrocarbon into syngas which is a mixture of carbon monoxide (CO) and H_2. It's an endothermic reaction and heat is often supplied from combustion of some of the hydrocarbon in the feed. The second step consists on a reaction called a "water gas shift" which converts CO produced in the first step into CO_2 and H_2 by reaction with H_2O (Riis *et al*., 2005, Tong & Matsumura, 2006). The process temperature and pressure vary respectively between 700 to 850 °C and 3 to 25 bar. This process has been developed at large scale and used for many years despite it being so energy intensive. The efficiency of steam reforming process is in the range of 65-75%. The only disadvantage is CO_2 emission into the atmosphere (Hemmes *et al*., 2003).

Partial oxidation of hydrocarbon. In this process, H_2 is produced through a catalytic partial combustion of hydrocarbon with pure O_2 gas (O_2). Carbon monoxide and H_2 are produced and then CO is further converted to CO_2 and H_2 by the "water gas shift" reaction as in steam reforming. This process is exothermic; there is no need for an external reactor heating system (Riis *et al*, 2005). The process efficiency is around 50%. It is also a process that releases CO_2 into the atmosphere (Hemmes *et al*., 2003).

Coal gasification. Syngas is produced by mixing coal with O_2 or air and steam in a reactor (fluidised bed, fixed bed or entrained flow). This process is comparable to the partial oxidation of hydrocarbon. H_2 is then produced by "water gas shift" reaction (Hemmes *et al*., 2003). Current research is on reducing the amount of CO_2 released by the process.

Electrolysis of water. Electrolysis of H_2O uses electrical energy to split H_2O into H_2 and O_2. This reaction is carried out by electron displacement between electrodes immersed in cells containing an electrolyte (H_2O mixed with some salt in order to enhance its conductivity) (LeRoy, 1983). This process is useful when highly pure H_2

is required by end users. The only problem is the availability of electricity which makes the H_2 produced expensive (Padró & Putsche, 1999, Kapdan & Kargi, 2006).

During electrolysis, it was found that the total energy demand for H_2O electrolysis increases slightly with temperature, while the electrical energy demand decreases. A high-temperature electrolysis process has been developed to favourably produce H_2. The process becomes efficient when high temperature is available as waste heat from other processes (Doenitz *et al*., 1980, Maskalick, 1986, Hemmes *et al*., 2003, Riis *et al*., 2005).

Biological H_2 production

Microorganisms are used to convert H_2O or organic materials into H_2 through two processes: photobiological H_2 production and dark fermentation.

Photobiological H_2 production. Microalgae and cyanobacteria (photoautrophic microorganisms) use radiation from light to split H_2O molecules into H_2 and O_2 by photosynthesis. Hydrogenase enzymes produced by microorganisms recombine protons and reductants (Ferredoxin and NADH: electrons donor) into H_2 gas molecules. Cyanobacteria can also fix nitrogen with nitrogenase enzymes as catalysts to produce H_2, but the activity of those enzymes is inhibited in the presence of nitrogen and O_2, therefore anaerobic conditions are required (Melis, 2002, Schütz, 2004, Dutta *et al*., 2005, Kapdan & Kargi, 2006).

Photoheterotrophic microorganisms are bacteria capable of converting organic acids (acetic, lactic, and butyric) as electrons donor into H_2 and CO_2 under anaerobic conditions in the presence of light. In this case the process is called photo–fermentation (Reith *et al*., 2003, Kapdan & Kargi, 2006)

Photobiological H_2 production may be considered the most economic process utilising simply H_2O, but it can only be operated during daytime. Also, production of O_2 from the process may decrease the H_2 efficiency by inhibiting the H_2O splitting reaction (Benemann, 1997, Das & Viziroğlu, 2001, Levin et al., 2004, Kovács et al., 2004, Kapdan & Kargi, 2006).

Dark Fermentation for H_2 production. This process utilises obligate and facultative anaerobic microorganisms to convert organic materials into H_2 from general anaerobic metabolism. The anaerobic production of H_2 involves the partial oxidation (in acidogenesis phase) of organic materials. Under natural anaerobic conditions the H_2 is used as an electron in the methanogenic reactions for the biochemical reduction of CO_2 to CH_4, as it is shown below (Figure 1) (Kovács *et al*., 2004). The anaerobic biohydrogen production process is not only stable, but also more rapid and it can be carried out in the absence of light compared to the photofermentation process (Lee *et al.*, 2004; Das & Veziroğlu, 2001).

More emphasis has been placed on the fermentative production of H_2 because it is renewable, environmentally friendly and less energy intensive. Other advantages of the fermentative H_2 production lie in the utilisation of waste materials. This process can couple H_2 production from various substrates in industrial and/or agricultural wastes to other forms of energy such as butanol and ethanol. Other end products of the process could also include high valued fine chemicals (Vessia, 2005, Gavrilescu, 2002, Fabiano & Perego, 2002, Sung *et al*., 2003, Kapdan & Kargi, 2006, Basak & Das, 2007).

1.2.3 Bioreactors used in H_2 production

Different types of bioreactor e.g. continuous stirred tank reactor (CSTR), upflow anaerobic sludge blanket (UASB) and the anaerobic FBBR have been used in the anaerobic treatment of wastewater for H_2 production. The choice of the bioreactors

depends on the strength of wastewater and they are mostly used in streams containing soluble organic wastes which could be converted by microorganisms in organic acids, alcohols and biogas (Murnleitner, 2001, Boe, 2006).

UASB and the anaerobic FBBRS are regarded as high rate bioreactors when compared to CSTR because of the ability of biomass retention facilitated by their designs. In these bioreactors, microorganisms are retained as immobilised bed (UASB) or attached to solids (anaerobic FBBR) in form of biofilm or granules and they are maintained suspended by the dragging force of the upward wastewater flow. This increases their catalytic activity and leads to high degradation rates of organic wastes. They can work at high hydraulic loading rates and also at low HRTs (Qureshi *et al*., 2005). When comparing the two bioreactors, the anaerobic FBBR is considered more efficient because of the fact that particle washout is less, making it more stable than the UASB. This is a reason why it has received more attention recently in wastewater treatment for H_2 production. A disadvantage of the anaerobic FBBR is the requirement of high energy to get fluidisation in the bioreactor (Marcoux, 1997, Reith *et al.*, 2003).

1.2.4 Biochemistry of anaerobic process

Anaerobic digestion as an organic waste treatment process uses microorganisms to degrade organic matter into small organic compounds such as long chain and volatile fatty acids (VFAs), alcohols and biogas. The process is carried out in the absence of O_2 in four successive stages. The catabolic and anabolic processes of metabolism of the different microorganisms in the anaerobic consortium are controlled by the process conditions such as temperature, pH, H_2 partial pressure, substrate loading rates and products removal. A schematic description of the anaerobic digestion process is presented below (Figure 1) (van Andel & Breure, 1984, Gavrilescu, 2002, Handajani, 2004, Luostarinen, 2005).

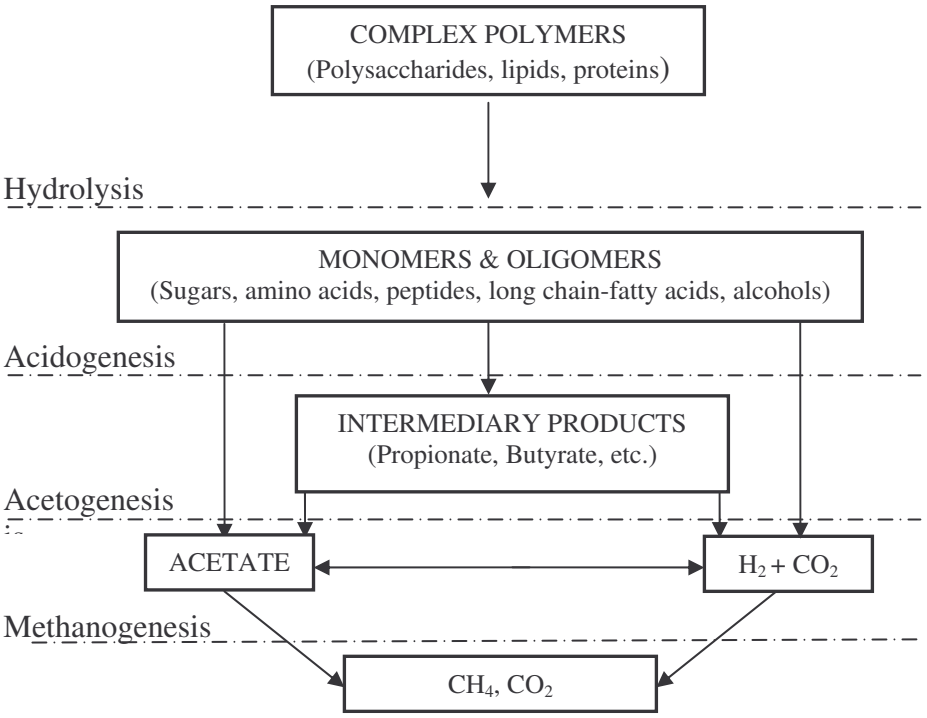

Figure 1 Different stages of anaerobic digestion of organic matter for CH_4 production.

Hydrolysis

Hydrolysis is the first step in the anaerobic process where complex organic materials or large biopolymers such as carbohydrates, proteins and lipids, which may be contained in wastewater, are broken down into simple soluble monomers, by incorporation of H_2O (Figure 1). These monomers which are the products of external hydrolytic reactions, can be taken up across cell membranes and used as substrates for catabolism and anabolism (Handajani, 2004, Luostarinen, 2005, Boe, 2006).

The breakdown of large biopolymers into the constituent monomers are catalysed by extracellular hydrolytic enzymes (cellulase, protease, lipase) released by facultative or obligate anaerobic bacteria (Gavrilescu, 2002). Depending on the type of anaerobic process (industrial or domestic wastewater treatment), hydrolysis is a rate-limiting

and temperature dependent step leading to the formation of sugars, amino acids, alcohols (Mahmoud, 2002, Handajani, 2004, Luostarinen, 2005, Boe, 2006, Mittal, 2006).

Acidogenesis

Acidogenesis, also called fermentation is a process by which soluble molecules are used as carbon and energy sources by fermentative bacteria and converted into VFAs, alcohols, and biogas (Reith *et al*., 2003). Acidogenesis is very important in anaerobic digestion as it is a step where H_2 is produced. H_2 comes from the mechanism of dehydrogenation of pyruvate by ferredoxin and NADH reductase enzymes and also from the conversion of formic acid by formate dehydrogenase (Oh *et al.,* 2002, Handajani, 2004).

H_2 is one of the substrates from which CH_4 is formed. If no H_2 were formed, fermentation could occur with electron exchange between organic compounds. This could yield a mixture of oxidised and reduced organic products lowering the energy level of the soluble matter and acidogenesis would not take place. For acidogenesis to take place, some conditions such as nature of the culture, temperature, pH and H_2 partial pressure must be controlled to direct the process to the formation of expected end-products (Gavrilescu, 2002).

Acetogenesis

Acetogenesis is part of the fermentation process where more reduced compounds such as aromatic compounds, long VFAs and alcohols are converted to acetic acid and H_2 (Figure 1) (Gavrilescu, 2002). This conversion is only possible if H_2 partial pressure is kept very low by H_2 uptake by methanogens. This oxidation is performed

by acetogen bacteria. This step is also dependant on the nature of the culture, pH and H_2 partial pressure.

Methanogenesis

This process involves methanogenic bacteria which convert H_2, acetate and CO_2 produced by the fermentation step to CH_4 and CO_2 as the final end products of anaerobic digestion of the macro biopolymers in wastewater (Figure 1). Two groups of methanogenic microorganisms are involved: acetate-oxidising methanogens, which can split acetic acid into CH_4 and CO_2 and H_2 oxidising methanogens, which reduce CO_2. Methanogenic bacteria are slow growing microorganisms which take place at low H_2 partial pressure and pH range 6-8 (Handajani, 2004).

1.2.5 Microorganisms involved in biohydrogen production

Many microorganisms have been identified as participating in the anaerobic wastewater treatment process. H_2 gas is synthesised by a large group of microorganisms that include both obligate and facultatively anaerobic bacteria. They can be classified into different functional groupings according to their temperature tolerance range as psychrophilic (13-18°C), mesophilic (25-40°C) or thermophilic (55-65°C) (Metcalf & Eddy, 2003). Growth of these bacteria depends on factors such as pH, nutrients and substrates which will be further elaborated on below (Metcalf & Eddy, 2003).

Cultures are selected either as single or multiple strains, especially for their adaptation to a substrate or raw material. When mixed cultures from activated sludge are involved in the anaerobic treatment process, an enrichment procedure for producing an inoculum suitable for biohydrogen production involves a heat shock and acidic pH treatments. The heat and acid pH treatments inhibit or kill non spore-forming bacteria which are H_2 consuming microorganisms (methanogens) and

enriches spore-forming bacteria (acidogens) (Hawkes *et al.*, 2002, Sung *et al.,* 2002). Species from Clostridia (obligate) and Enterobateriaceae (facultative) families have been widely used in biohydrogen production.

Clostridium species

Microorganisms of the *Clostridium* genus have been identified in many anaerobic bioremediation treatment processes. They are rod-shaped, gram-positive, spore forming and obligate anaerobes. In biological H_2 production, they have been found to use the pyruvate-ferredoxin oxido-reductase pathway with H_2, acetate, butyrate, acetone, butanol and ethanol as end products. The only disadvantage of clostridia species is their vulnerability to inhibition by O_2 (Hawkes *et al.,* 2002). *Clostridium pasteurianum*, *Clostridium butyricum*, *Clostridium cellulolyticum*, *Clostridium thermolacticum* are some of bacteria which have been used successfully in biological H_2 production (Collet *et al*., 2004, Lin & Lay, 2004a, Chang *et al*., 2006).

Enterobacter species

Microorganisms of the Enterobacteriaceae family are facultative anaerobes, gram-negative, rod shaped and recognised as glucose fermenters and nitrate reducers. They utilise a wide range of carbon sources. They have been used in many studies and found to be following the butanediol fermentative pathway with mixed acid products (Hwang *et al.,* 2004). H_2 is produced via the ferredoxin oxido-reductase system. *Enterobacter cloacae, Enterobacter aerogenes*, *Citrobacter freundii*, *Citrobacter intermedius* have been used in some experiments and they conducted to a high yield of H_2. Along with H_2 the following were also produced including acetate, ethanol, 2,3-butanediol, acetone, ethanol and of course CO_2 (Rachman *et al.,* 1998, Jung *et al.*, 1999, Palazzi *et al.*, 2000, Thompson, 2005).

1.2.6 Effect of Fermentative parameters

Anaerobic digestion is a biological process which is influenced by environmental factors such as temperature, pH, H_2 partial pressure, feedstock (carbohydrates) and other soluble metabolites formed.

Temperature

The efficiency of bioprocesses is temperature-dependent due to the strong reliance of chemical (biochemical) reactions such as enzymatic activity and cellular maintenance upon temperature (Luostarinen, 2005). Temperature is one of the most important factors affecting other parameters such as pH, oxido-reduction potential, electrons transfer; which activate the degradation of organic matter, the rate of microbial growth, and consequently the rate of metabolites formation and biogas production in an anaerobic digestion process (Seghezzo, 2004, Zhang & Shen, 2006). The effect of different temperatures on growth rate could be predicted in terms of the activation energy required for growth as in enzyme-catalysed chemical reactions (Metcalf & Eddy, 2003).

An optimum temperature exists at which each micro-organism can survive and grow depending on whether the microbes are psychrophilic, mesophilic or thermophilic. Above the optimum temperature for the specific group, cell degradation can become probably dominant over growth processes. With temperature below the optimum, cell growth can proceed slowly or not at all because the cell membrane is not fluid to be penetrated by nutrients needed for growth (Murnleitner, 2001). The range of temperatures has also an implication on the type of biochemical reactions taking place in the bioreactor (Seghezzo, 2004, Zhang & Shen, 2006).

Most of biohydrogen production by anaerobic processes operate at ambient temperature (30-40°C) with the advantage of being efficient and less energy intensive

(Seghezzo, 2004, Zhang & Shen, 2006). However, some research has been carried out at low (psychrophilic) and high temperatures (thermophilic and hyper thermophilic ranges) to study the influence of the environmental temperature change on the anaerobic treatment processes.

In wastewater treatment the psychrophilic process is rarely used because of the slower degradation rates of substrates due to slow growth of microorganisms (Seghezzo, 2004). At low temperatures the process tends to be slow and thus requires long hydraulic and solid retention times, resulting in large reactor volume which may not be economically viable (Luostarinen, 2005). However, low temperature fermentation processes can be used for treatment of low-strength wastewater (Rebac *et al*., 1999)

Thermophilic processes for biological H_2 production have been studied and were successful especially when pathogens had to be removed from the liquid organic materials and in the case of wastewaters containing high strength organic matter (Hawkes *et al*, 2007). High temperatures are known to encourage the growth of suspended biosolids and increase biochemical reactions. Consequently, operations are performed at high nutrient loading rates which lead to high products formation and better process efficiency (Kotsopoulos *et al*, 2005, Zurawski *et al*, 2005). The energy required to maintain high temperature is the only economic problem (Liu & Tay, 2004, Hawkes *et al*., 2007).

pH

pH has a significant impact on the performance of anaerobic processes in wastewater treatment. It determines the degradation pathway of organic matter and has an effect on microbial activities as in biochemical operations (Horiuchi *et al*, 2002, Hwang *et al.,* 2004, Boe, 2006). Microorganisms have an optimum pH value from which any deviation can cause change in their behaviour. pH can be maintained at its optimal

range by addition of sufficient buffers like bicarbonates (Liu & Tay, 2004, Seereeram, 2004, Boe, 2006).

In biological H_2 production, degradation of organic material contained in wastewater is performed as presented in the above chapter. A pH between 6.0 and 7.4 has been found as acceptable for the activity of the hydrolytic microorganisms (Liu & Tay, 2004, Luostarinen, 2005, Boe, 2006). Many studies reported that the acid-producing bacteria, which are responsible for H_2 fermentation processes have an optimal pH range of 5.0 to 6.0 (van Ginkel *at al*, 2001, Oh S. *et al*., 2003, Khanal *et al*., 2004, Liu & Tay, 2004, Kawagoshi *et al*., 2005, Nath *et al*.,2005, Boe, 2006, Kapdan & Kargı, 2006, Hawkes *et al*., 2007, Venkata *et al.,* 2007). At this range, an increase on the production of VFAs, particularly acetic acid, butyric acid and propionic acid has been observed (Hawkes *et al*., 2007, Venkata *et al.,* 2007). These soluble metabolites determine the pathway which enhances the H_2 production (Nath *et al*, 2005). Similarly, a pH of 5.5 has been found to be optimal for high H_2 production rate and high H_2 yield (Sung *et al*., 2002, Sung *et al*., 2003, Fan *et al*., 2004, Fan *et al*., 2006, Hawkes *et al*., 2007).

An increase above this range to pH 8.0 tends to favour the growth of methanogens which inhibit the growth of acidogenic bacteria, lowering the H_2 production (Handajani, 2004, Liu & Tay, 2004, Seghezzo, 2004, Leitão *et al*., 2006). A lower pH to 4.5, shifts the VFAs-producing pathway to an alcohol-producing pathway which lowers the H_2 yield (Levin *et al*., 2004, Zhang & Shen, 2006).

H_2 partial pressure and soluble metabolites

H_2 partial pressure plays an important role in anaerobic digestion process. It has a direct effect on the proportion of the various intermediate products of the anaerobic reactions (Cord-Ruwisch *et al*., 1997, Schink, 1997, Leitão *et al*., 2006).

As presented above, fermentative H_2 production by anaerobic process is a partial oxidation of organic materials in CH_4 production process. During the anaerobic fermentation the hydrogenase reaction, involving enzyme-catalysed transfer of electrons from an intracellular electron carrier molecule to protons, is thermodynamically unfavourable and depends on the range of H_2 partial pressure (Angenent *et al*., 2004, van Ginkel & Logan, 2005, Kim *et al*., 2006a, Mandal *et al*., 2006).

$$pH_2, max \leq \exp[2F(E^{'\circ}_{H_2} - E^{'\circ}_{x})/ RT]$$

Where $E^{'\circ}_{x}$ is the redox potential of the electron donor, F is the faraday's constant, R is the ideal gas constant, and T is the absolute temperature (Angenent *et al*., 2004, Mandal *et al.,* 2006).

The transfer of electrons from the electron donating carbon skeletons to inorganic electron acceptors such as protons, in the liquid phase is facilitated by the electron carriers such as nicotinamide adenine dinucleotide (NADH, $E^{'\circ}_{NADH}$ = -320 mV) and ferredoxin (Fd, $E^{'\circ}_{Fd}$ = -400 mV). With the redox potential of the proton/dihydrogen couple $E^{'\circ}_{H_2}$ = -414 mV, H_2 partial pressures have to be lower than 40 Pa (0.3 atm) or 60 Pa ($6x10^{-4}$) to allow electrons to be released as molecular H_2 from NADH or ferredoxin. Consequently, a low H_2 partial pressure promotes H_2 generation with production of acetate and CO_2 as co-products rather than ethanol or butyrate (van Andel & Breure, 1984, Schink, 1997, Angenent *et al*., 2004, Boe, 2006). In contrast, high H_2 partial pressures stimulate the accumulation of propionate, reduced fatty acid compounds and alcohols in the liquid phase with decrease in the H_2 production rate and H_2 yield (Luostarinen, 2005, van Ginkel & Logan, 2005). Therefore, the H_2 partial pressure has to be maintained at a low level to allow H_2 synthesis during a continuous fermentation process. It is only in high temperature systems that H_2

partial pressure does have little effect on bacterium metabolism in the biological H_2 production process (Levin *et al*., 2004).

Addition of KOH in the liquid phase, sparging of gas such as N_2 or CO_2 into the head space of the bioreactor and also removal of H_2 when produced are some of the methods used to reduce H_2 partial pressure in order to increase H_2 production rate and H_2 yield (Hawkes *et al*., 2002, Göttel *et al*., 2005, Kim *et al*., 2006a, Mandal *et al*., 2006).

Biological H_2 production is usually accompanied by soluble metabolites production (VFAs and solvent). The production of these intermediate products reflects changes in the metabolic pathway of the microorganisms involved. A better knowledge of such changes could improve the understanding of conditions favourable for H_2 production (Khanal *et al.*, 2004, Levin *et al*., 2004, Zhang & Shen, 2006).

The major VFAs detected are acetate, butyrate, propionate, succinate, lactate and formate (van Andel & Breure, 1984). The first three VFAs are the most commonly found in biological H_2 production and used to assess the process performance (Mösche & Jördening, 1998, Handajani, 2004, Kapdan & Kargi, 2006). Theoretically 4 moles and 2 moles of H_2 gas can be generated from a mole of hexose when acetic and butyric acids are end-products respectively. Thus high H_2 yields are associated with a mixture of acetate and butyrate fermentation products (Levin *et al.*, 2004). Propionate production is a H_2 dependant pathway (it consumes H_2 when present into the reactor) (Hawkes *et al*, 2002, Hawkes *et al*., 2007). Preventing the initiation of this pathway will help to increase the H_2 production rate (Vavilin *et al.*, 1995, Hawkes *et al*, 2002, Levin *et* al., 2004, Lin *et al*., 2006).

Mostly ethanol, butanol, butanediol, acetone accompany VFAs formation during anaerobic H_2 production. It is known that the accumulation of alcohol into the bioreactor decreases the H_2 production rate and H_2 yield (Hawkes *et al*., 2002, Reith

el al., 2003). This is due to the fact that reduced fermentation end-products containing H_2 which has not been liberated as H_2 gas and also electron donors produced during fermentation processes (important for hydrogenase enzymes), are mostly consumed by these products (Ueno *et al*., 2001, Levin *et al*., 2004). Therefore, to maximise H_2 yield, bacterial metabolism during fermentation process must be directed away from alcohols and reduced acids formation towards VFAs (Hawkes *et al*., 2002, Hwang *et al.,* 2004, Levin *et al*., 2004).

Temperature, pH, H_2 partial pressures and HRT are parameters which can regulate the preferred metabolites formation pathway leading to high H_2 production and H_2 yield (Gavrilescu, 2002).

CHAPTER TWO - MATERIALS AND METHODS

2.1 Seed inoculum

For the first experiment concerning the biological H_2 production in the anaerobic FBBR using two bacteria species, the seed inoculum was composed of facultatively anaerobic bacteria: *Citrobacter freundii* (Cf1) and *Enterobacter cloacae* (Ecl). Cf1 was obtained from sewage sludge samples (Olifantsvlei Municipal Sewage Treatment Plant, Johannesburg, South Africa). Ecl was isolated from samples taken from gardens at the University of the Witwatersrand (Johannesburg, South Africa). They were identified and characterised (Thompson *et al*., 2006; Thompson *et al*., 2008).

In the second experiment a mixed culture of undefined bacteria was obtained from an anaerobic primary sewage sludge sample collected from Olifantsvlei Municipal Sewage Treatment Plant (Johannesburg, South Africa). This culture was used as the bioreactor seed inoculum. Removal of possible pathogens and methanogens from the seed sludge was performed by the following treatment. Twenty-five mL of liquid sludge sample was preheated at 96°C for 20 minutes and incubated overnight at 37°C in nutrient broth (NB). Five mL was removed from the sample and heated at 60°C overnight after its transfer to a fresh 25mL of NB. Finally 5mL of the heated inoculum was adjusted to pH 5.0 by 0.1 N HCL and incubated overnight at 37°C. This final volume was conserved in NB and served as stock to seed the anaerobic FBBR inoculum.

2.2 Culture medium

The culture medium used in the bioreactor was a modification of the Endo formulation (Endo *et al*., 1982). The modification involved changing the C:N:P ratio of the original 334:42:1 to 334:28:5.6 (Thompson *et al*., 2006). Inorganic nutrient components of the medium consisted of (in g/L): NH_4HCO_3 3.464, $NaHCO_3$ 6.72,

K_2HPO_4 0.692, $MgCl_2.6H_2O$ 0.1, $FeSO_4.7H_2O$ 0.025, $CuSO_4.5H_2O$ 0.005, $CoCl_2$ $1.24x10^{-4}$ (Appendix 3). While nutrient concentration was kept constant the nutrient load into the anaerobic FBBR depended on the HRTs.

Sucrose was used as the organic carbon source. The sucrose was supplied to the bioreactor at a concentration of 17.65g/L which corresponds to a COD of 20g COD/L. Depending on the HRT the sucrose loading rate or the organic loading rate (OLR) ranged from 10 - 40 gCOD/L. The impact of the OLR on H_2 production was monitored.

2.3 Carrier material

Two carrier materials were used for biofilm and bacterial granules formation:

Initially sand, with a particle diameter ranging from 0.6 to 1.1mm was used as bed in the anaerobic FBBR. Prior to its use, sand particulates were first washed with distilled H_2O to remove all suspended fine colloidal particles and then autoclaved for 20 minutes to kill any microbial contaminants.

Then, irregular granular activated carbon (GAC) with the same particle size as sand and subjected to the same pre-treatment as described above, was used to replace sand in the anaerobic FBBR as carrier matrix for microorganism growth leading to biofilm and bacterial granules formation.

2.4 Experimental set-up

A schematic diagram of the experimental setup is shown in Figure 2 (Appendix 1). The anaerobic FBBR consisted of a clear Perspex cylinder (internal diameter (ID): 70 mm; wall thickness: 5 mm; height (H): 1000 mm) connected to an upper section with an expanded diameter (ID: 140 mm and H: 200 mm) for solid-liquid separation. The

bottom section of the anaerobic FBBR consisted of a conical shaped diffuser (ID: 70 mm and H: 150 mm) as the primary inlet. A stainless steel sieve was placed above the diffuser. The sieve was covered with a 30 mm layer of 5 mm glass beads (Marcoux, 1997, Schreyer & Coughlin, 1999, Zhang *et al*., 2007). The anaerobic FBBR had a working volume of 7.0 L and contained GAC with a settled volume that occupied 30% of its total volume. To reduce channelling at the bottom of the GAC bed 4 additional inlets positioned at right angles were placed above the glass bead layer at the base of the anaerobic FBBR. The effluent from the anaerobic FBBR was first passed through a 1 L Perspex cylindrical chamber housing the monitoring probes after which it flowed into the upper end of the gas-liquid disengager device. The effluent exiting from the bottom of the liquid-gas exchanger was collected into a 4.0 L reservoir containing two outlets. The upper outlet was used to drain away the excess effluent. The lower outlet was connected to a variable Boyser AMP-16 peristaltic pump (Boyser, Italy). This pump was used to recycle the balance of the effluent back into the anaerobic FBBR at a flowrate of 1.5 L/min in order to expand the GAC bed and maintain it in state of porosity greater than 80%. The probe chamber contained the thermocouple, pH, ORP and conductivity probes. These probes were linked to a data-taker which monitored measured data in digital mode according to a program loaded onto the computer. The total system volume; bioreactor, probe chamber, reservoir and tubing; was approximately 13 L. The operating temperature of the anaerobic FBBR was maintained at 37±1°C by means of a surrounding water-jacket which was connected to a waterbath. The bioreactor pH was kept between pH 5.5-6.0 controlled automatically using 3M HCl and 6M NaOH for acid or base adjustments via respective peristaltic pumps.

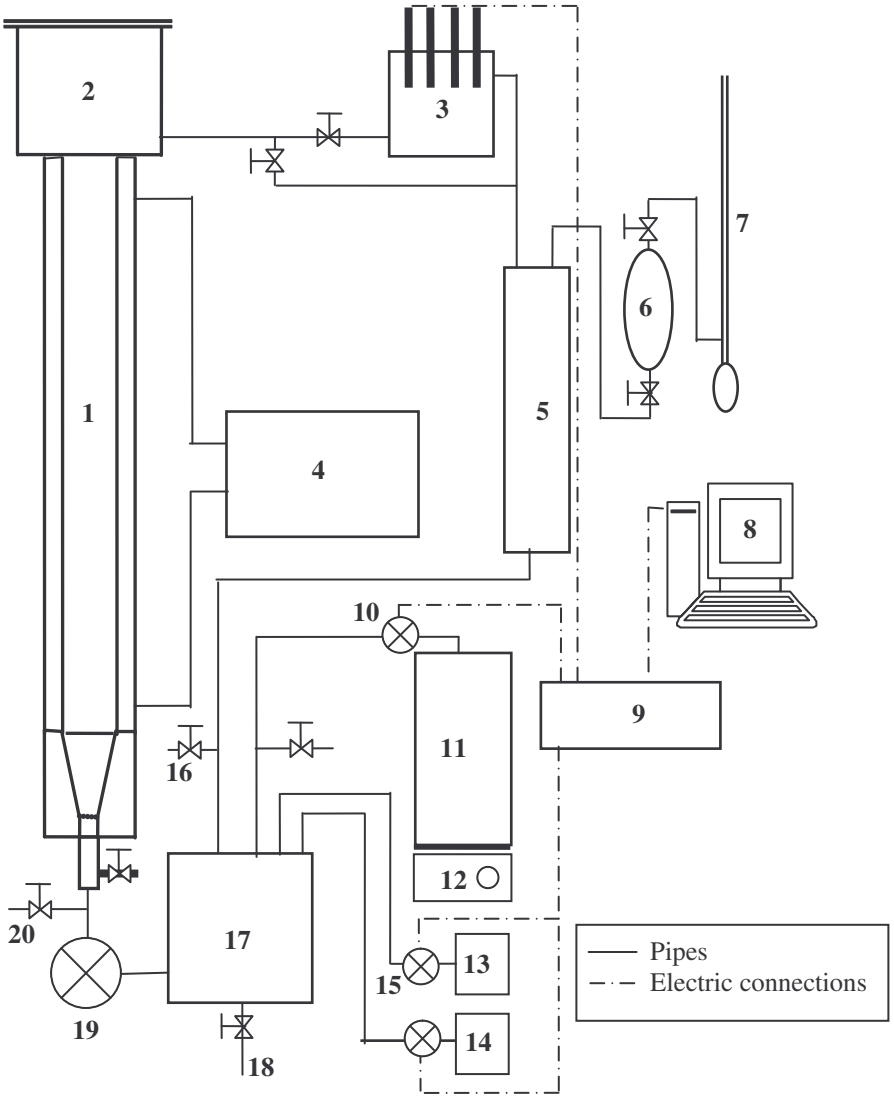

Figure 2 Scheme of anaerobic Fluidised Bed Bioreactor.

1 – Anaerobic FBBR, 2 – Solid-liquid separator, 3 – Electrodes box, 4 – Waterbath reservoir, 5 – Liquid-gas separator (Gas exchanger), 6 – gas bomb, 7 – Bubble meter, 8 – Computer, 9 – Data logger, 10 – Feed pump, 11 – Feed reservoir, 12 – Magnetic stirrer, 13 – HCL reservoir, 14 – NaOH reservoir, 15 – Dosing pump, 16 – Sampling port, 17 – Recycle reservoir, 18 – Overflow outlet, 19 – Recycle pump, 20 – Sampling port

2.5 Start up of the anaerobic FBBR

Bioreactor seed inoculum consisting of either an overnight culture of Ecl and Cf1, or treated sewage inoculum, was pumped into the anaerobic FBBR. Following the inoculation the anaerobic FBBR was operated on a batch-recycle mode for 3 days to acclimatise the bacteria and allow for their attachment to the GAC. After the three days the bioreactor operation was switched to continuous – recycle mode with an initial HRT of 8 h. The HRT was then decreased by increasing the medium supply rate. As the HRT was decreased from 8 to 4 h the growth and development of bacterial biofilm on the carrier became visible. With further decreases in the HRT below 4h the biofilm growth increased resulting in the initiation, development and growth of bacterial granules at the upper surface of the expanded GAC bed. An expanded granular bed grew with increasing production of bacterial granules. Full fluidisation of the granular bed occurred once the HRT was decreased below 4 h. After formation of bacterial granular bed the HRT was decreased stepwise to a minimum of 0.5 h.

2.6 Anaerobic FBBR parameters monitoring

The bioreactor's pH, ORP, conductivity and internal bioreactor temperature were monitored continuously. Sucrose and COD consumptions, biogas production, soluble volatile fatty acids (VFAs), and volatile suspended solids (VSS) concentrations were measured during the course of experiments. All measurements were carried out under quasi-steady state conditions with regard to the operation of the anaerobic FBBR. These quasi-steady state conditions were defined as ones under which variations in the above parameter values were small (less than ±10% variation) during a set of analysis (Zhang *et al.,* 2007). The quasi-steady state conditions were confirmed by the stability of optical density (OD) measured using a spectrophotometer at the optimum wavelength of 520 nm. All measurements have been given as averages of a minimum of three replicates.

2.7 Analytical methods

2.7.1 Substrate measurements

Sucrose concentrations in the influent and the effluent of the reactor were determined colorimetrically using the resorcinol method (Kerr *et al.* 1984). Prior to analysis, a standard curve was made in the range of 0-10mM using pure sucrose.

Determination of dissolved chemical oxygen demand (COD) was carried out as follows: 10 mL of bioreactor sample was centrifuged at 10 000 rpm for 15 min., 0.2 mL of the supernatant was removed from the sample and digested in the Hanna digesting block C 9800 preheated at 150°C according to the procedure described in the Hanna catalogue (Hanna instruments, USA), using the Hanna closed reflux reagent vials for sample digestion. A Hanna C 214 Multiparameter Bench Photometer for wastewater treatment application was set at 610 nm wavelength and used for COD measurements. COD concentrations of samples were measured against a blank made with distilled H_2O as reference.

2.7.2 Biogas measurements

The volumetric rate of biogas production was measured with a bubble meter. For gas content analysis, gas was collected in a glass bomb connected in series with the bubble meter. H_2, CO_2 and CH_4 compositions were quantified using a Pye Unicam (Gomac) gas chromatograph (GC) equipped with a thermal conductivity detector (TCD). The operational temperatures of the injection port, column and detector were 95°C, 80°C and 100°C, respectively. Helium was the carrier gas set at a flowrate of 30mL/min and the column was packed with Porapak Q (80/100 mesh, Supelco, South Africa).

2.7.3 Soluble metabolite measurements

VFAs such acetic, butyric and propionic acids were detected by a HP5890 GC equipped with a flame ionisation detector (FID) and a Nukol column (Supelco, South Africa). Before performing any liquid measurements, samples were subjected to a filtration using a 0.22 µm membrane filters, then 1 µL of sample was injected onto the GC column using a syringe. The following GC conditions were used: carrier gas, Helium; flowrate, 30 mL/min; injector temperature, 90°C and detector temperature, 250°C. The initial oven temperature was 40°C. This temperature was maintained for 5min, then ramped to 190°C at 10°C/min and held for 10 min before returning to 40°C. Prior to VFA analysis standards were run for characterisation and quantification.

2.7.4 Bacterial and VSS measurements

Monitoring of non-attached planktonic bacterial densities was carried out by serial dilution plate counting of overnight colonies that had been incubated on nutrient agar (NA) at 37°C (Lindsay & von Holy, 1999). For the monitoring of attached bacteria, 1 g of GAC was removed from the bioreactor bed and shaken for 10 min in Schott bottles filled with 20g of glass beads (5 mm in diameter) and 10 mL of sterile H_2O. After leaving to stand for a further 10 min, an aliquot of the supernatant was serially diluted before 1 ml samples were spread onto NA plates. Colony-counts for the attached bacterial were carried out after overnight incubation of at 37°C (Lindsay & von Holy, 1999).

For estimation of biomass production, the concentration of VSS was determined by passing 10 mL of sample through 0.22 µm membrane filters. The residue collected on the filter was dried in an oven at 105°C for 24 hours and measured according to the procedures described in standard methods (American Public Health Association, 1998).

2.7.5 Scanning electron microscopy analysis

Morphological studies of immobilised cells in biofilms were performed with a scanning electron microscope (SEM, JEOL JSM-840, Japan) at 20 kV and a working distance of 15 mm. One gram of each biofilm sample was washed with sterile H_2O to remove all unattached bacteria and fixed for 24 h in 3 % glutaraldehyde, followed by dehydration in a graduated series of ethanol (10 – 100%) for 10 minutes each step. The pre-treated sample was then critical-point dried and coated with gold-palladium splutter for SEM observation (Lindsay & von Holy, 1997).

2.7.6 Bacteria Identification

To confirm the identities of bacteria in each bioreactor setup, bacterial samples from the bioreactor were prepared for DNA extraction by the streak plate method. After overnight growth at 37°C isolated single colonies were selected for DNA extraction according to the InstaGene® procedure (Biorad, South Africa). A polymerase chain reaction (PCR) amplification was performed to amplify the 16S ribosomal deoxyribonucleic acid (16S rDNA) gene of isolates. The primer set used for the amplification of 16S rDNA were 27f (5'-AGA GTT TGA TCM TGG CTC-3') for the forward primers and 1392r (5'-ACG GGC GGT GTG TRC-3') for the reverse primers (Collins *et al*., 2005, Yang *et al*., 2007). For the amplification of the target template DNA, the PCR reaction mixture consisted of as per manufacturer instructions: 1 µL each of forward and reverse primers, 20 µL of the DNA template, 3 µL of sterile H_2O, 25 µL of 2X PCR Master Mix (Fermentas Life Sciences, www.fermentas.com) to give a final volume of 50 µl. The PCR reaction was carried out in a thermocycler (Applied Biosystems/Gene Amp®PCR System 2700). PCR amplifications were performed using the following conditions: initial denaturation of template DNA at 95°C for 3 min; followed by 35 cycles of: template denaturation at 94°C for 30s, primer annealing at 60°C for 45s, primer extension for1min 30s at 72°C, and a final extension reaction at 72°C for 7 min.

The PCR products were separated in 1% (w/v) agarose gels (Sigma) stained with ethidium bomide in electrophoresis 5 X TBE-buffer (89 mM Tris, 89 mM boric acid, 2mM sodium EDTA × 2 H_2O, final pH 8.3) at 80V. Five microlitre of the PCR products were mixed with 1µl of tracking dye (Promega) then the mixture was loaded into the gels. Six microlitre of 1 Kbp DNA ladder (Promega) was loaded into the gels and used as standard. The separated DNA bands in the agarose gels were viewed under ultraviolet light (UVP GelDoc, Biorad).

Purified PCR products were sequenced by Inqaba Biotechnical Industries (Pty) Ltd (P.O. Box 14356, Hatfield 0028, South Africa) and the sequences were analysed by NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) by aligning against the 16S rDNA sequences from GenBank (GenBank database of the National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/GenBank/).

It has to be noted that this procedure has been performed on the two isolates (*Citrobacter freundii* and *Enterobacter cloacae*) prior to their use in the first experiment and a phylogenetic tree highlighting the clustering of the isolates was constructed using the neighbour joining method and bootstrapping in DNAMAN version 4 (Lynnon Biosoft) with E.coli used as outgroup (Lindsay *et al*., 2008).

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2.8 Statistical analyses

The mean and standard deviation of data were calculated using the AVERAGE and STDEV functions of Excel worksheet from OriginLab© software program (version 6.1).

CHAPTER THREE – BIOHYROGEN PRODUCTION BY CARRIER INDUCED GRANULES

3.1 Performance of the anaerobic FBBR for H_2 production using two strains (Cf1 and Ecl)

3.1.1 Carrier induced granular sludge beds

Recently high rates of biohydrogen production (7.4 L/(L.h)) have been achieved in bioreactors containing a fluidised bed consisting of self-flocculated anaerobic granular sludge (Lee *et al*., 2003; Lee *et al.*, 2004; Lee *et al.*, 2006a, Lee *et al*., 2006b). They have described their system as a carrier induced granular bed (CIGB) bioreactor for anaerobic biohydrogen production (Lee *et al.*, 2006b). Furthermore, Lee *et al* (2003) have noted that before their work only two other reports have described the application of self-flocculated cells (Rachman *et al*., 1998) or granular sludge (Fang & Liu, 2002) for anaerobic H_2 production. It was discovered that granule formation was significantly stimulated by packing a small quantity of carrier media such as GAC into the bottom of the upflow bioreactor (Lee *et al*., 2004). Several types of carrier media were tested (CAC: cylindrical activated carbon; SAC: spherical activated carbon; SD: sand; FS filter sponge). Maximum H_2 production rates (6.8 to 7.3 L/(L.h)) were achieved for CAC and SAC carriers, whereas the rates (3.4 to 3.8 L/(L.h)) obtained for FS and SD carriers were substantially lower (Lee *et al*., 2004). While it was not made clear how the CAC carriers induced the growth and development of granules or stimulated the occurrence of self-flocculation of cells; it was observed that granule formation only occurred under conditions when the CAC bed porosity was greater than 90%, which in turn required a hydraulic retention time (HRT) of less than 4 h (Lee *et al*., 2003). A similar observation has been reported by Thompson *et al*. (2008) where granule formation did not occur for settled beds of granulated activated carbon (GAC), which in turn corresponded to flow rates equal to 1 L/min for a bioreactor with the following dimensions: internal diameter of 80 mm

and height of 1000 mm. However when the flow rate for the bioreactor was increased to above 1 L/min the settled bed of GAC expanded and granule formation occurred within 48 h.

Nucleotide sequence accession numbers and phylogenetic trees

Figures 3 and 4 represent phylogenetic trees of *Citrobacter freundii* and *Enterobacter cloacae* based on their 16S rDNA and constructed using the neighbour joining method and bootstrapping in DNAMAN version 4 (Lynnon Biosoft).

DNA extraction was performed on the two strains: *Citrobacter freundii* (Cf1) and *Enterobacter cloacae* (Ecl.) prior to their use as inoculum in the anaerobic FBBR for H_2 production. Their 16S rDNA sequences have been submitted into GenBank database and assigned the following accession numbers: *Citrobacter freundii* (EU046372) and *Enterobacter cloacae* (EU046373); thereafter a phylogenetic tree of isolates was constructed with strains taken from BLAST results on NCBI. Figures 3 and figure 4 showed similarities between our isolates and illustrate that Cf1 and Ecl strains used in this study were similar to other isolated strains of Cf1 and Ecl both known as genius from Enterobacteriaceae family.

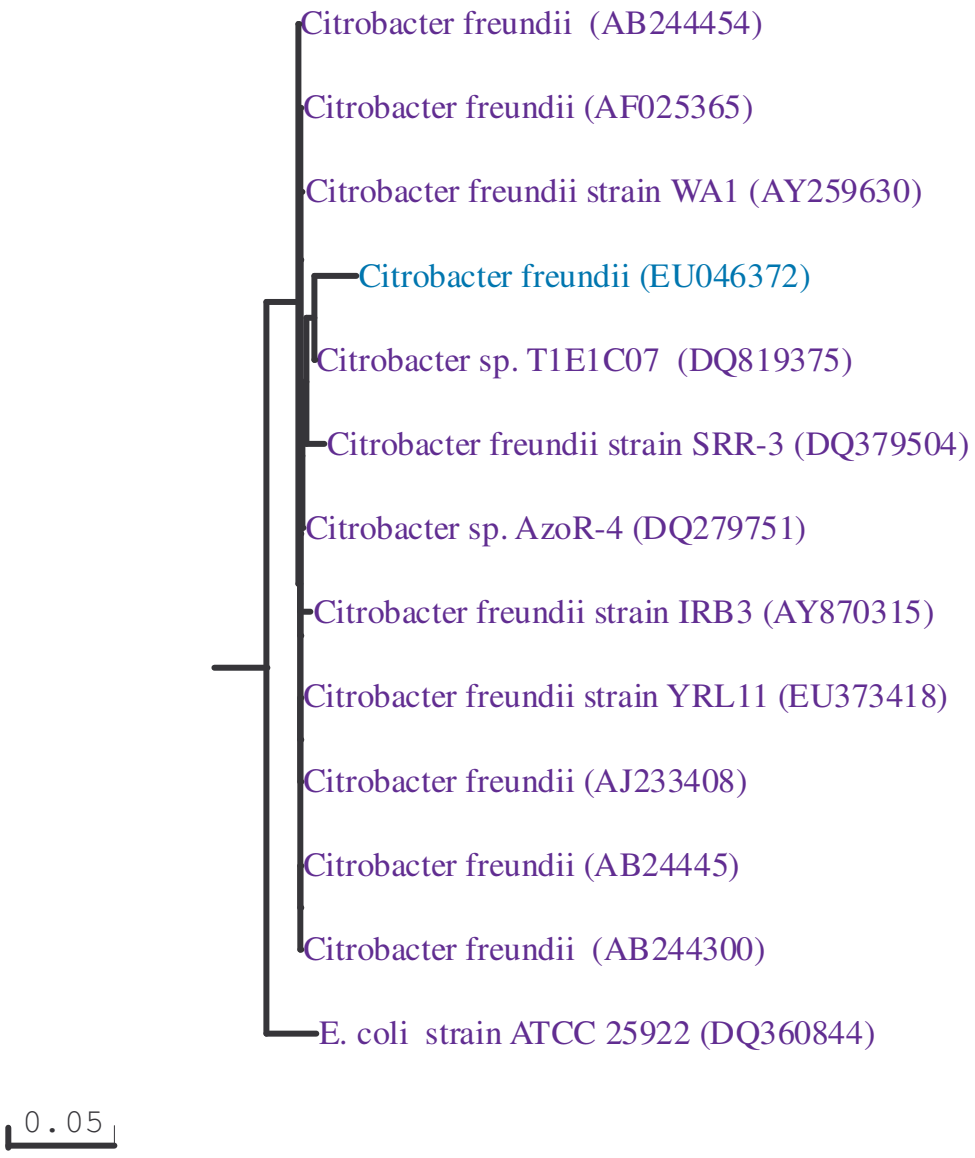

Figure 3 A phylogenetic tree of *Citrobacter freundii* (light blue) constructed using the neighbour joining method and bootstrapping in DNAMAN version 4 (Lynnon Biosoft) with E. Coli used as the outgroup. The bar indicates the difference of 5 nucleotides par 100

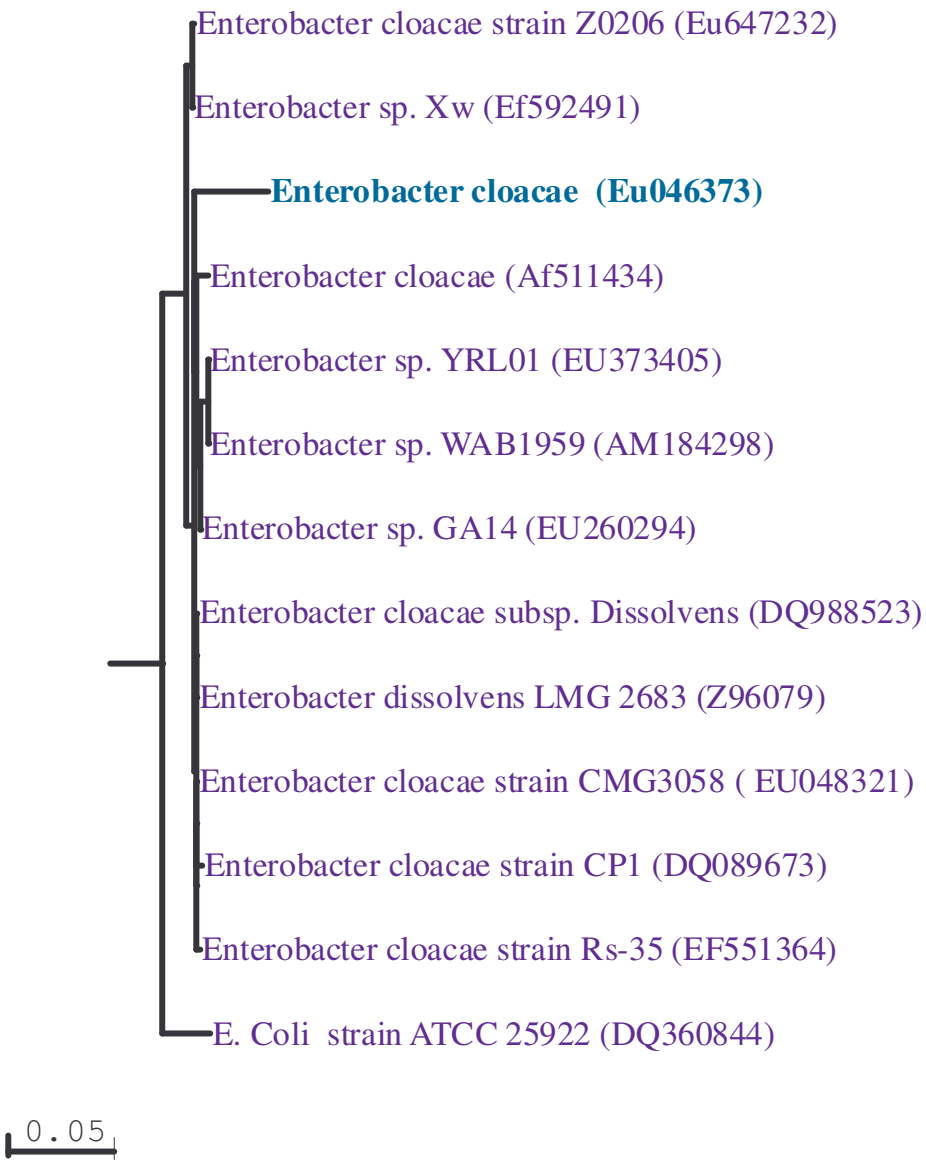

Figure 4 A phylogenetic tree of *Enterobacter cloacae* (in bold) constructed using the neighbour joining method and bootstrapping in DNAMAN version 4 (Lynnon Biosoft) with E. Coli used as the outgroup. The bar indicates the difference of 5 nucleotides par 100

Use of sand

Preliminarily, experiments were carried out using sand as the carrier matrix into the anaerobic FBBR (Results not shown).

The anaerobic FBBR was filled with sand at 30% (v/v) of its total volume as the bioreactor bed, and then inoculated with Ecl and Cf1. The anaerobic FBBR start up was performed as described in materials and methods. Bacterial growth took place in the anaerobic FBBR with formation of attached layers of biofilm to sand. After more than 30 days of running time, it was observed that biofilm was not growing and carrier induced granular sludge formation was not taking place; consequently the bioreactor bed was not expanding. Planktonic cell counts were 7.0 log cfu/mL and attached cells showed a constant value of 3.0 log cfu/g. It was decided to increase OLR by decreasing HRT with hope of enhancing nutrient availability in the anaerobic FBBR. Only planktonic cell counts increased to 9.0 log cfu/mL and remained constant at that value without any observed change in attached cells.

A plate of NA with colonies grown overnight showed the presence of two distinctive cells which were assumed to be Ecl and Cf1 used as inoculum. This confirmed that contaminants were not present into the anaerobic FBBR. Measurements of OD of the bioreactor liquid showed an increase due to planktonic cells growth only (from 2.1 to 2.6) followed by a stationary phase corresponding to the above-mentioned value of cell counts.

The system was comparable to a fixed bed even if the bed was maintained suspended by the incoming media and recycle flowrates (which were changed above 1L/min with hope of expanding the bioreactor bed). Biogas production rate was unstable and fluctuated around 0.96 L/(h.L). The H_2 and CO_2 contents in the biogas were measured about 36% (v/v) and 23.3% (v/v) respectively. Any change in HRTs did not imply change in biogas production as planktonic cells were washed out from the anaerobic

FBBR due to a decrease of HRT. An analysis of the anaerobic FBBR liquid showed that only butyric and lactic acids were detected at 133.3±6 and 101±10 mg/L respectively. This explained the value of biogas production rate which was related to the butyrate pathway.

As a packed bed, the interstice between particles in the anaerobic FBBR was leading to detachment of biofilm layers from the carrier matrix due to the sand density and to high friction of particles. Another factor influencing the non expansion of the bed could be due to the irregular shape and smoother surfaces of sand. This led to a thin layer of biofilm attached and also explained less resistance of biofilm to friction. It was decided to shift to the use of GAC as carrier matrix.

Use of GAC

When the recycle flow rates were equal to or less than 1 L/min no visible biofilm or granule growth occurred with decreasing HRTs and the associated increases in the organic loading rates. At these recycle flow rates the GAC bed remained in a settled state. It appears that under nutrient non-limiting conditions carrier induced granulation does not readily take place in a settled bed of GAC. However, for all HRTs or organic loading rates, biofilm growth leading to granulation was initiated within 24 h, when the bed was expanded by increasing the recycle flow rates to a level greater than 1 L/min. With recycle rates of 1.5 L/min growth and development of granules readily occurred within 48 h after the HRT was reduced to below 4 h (Figure 5). As the HRT was further decreased stepwise to 0.5h, with the recycle rate remaining at 1.5 L/min, the granules increased in diameter from 3 to 5 mm (Figure 6). The bed expanded at 100% of the anaerobic FBBR volume with a rate of 2.1% per hour. The expansion (ε) was calculated in terms of height variation of the bed as the area of the bioreactor cross section was constant.

$\varepsilon = [(H - H_o)/H_o] \times 100\%$

Where H is the average bed height, H_o is the static bed height.

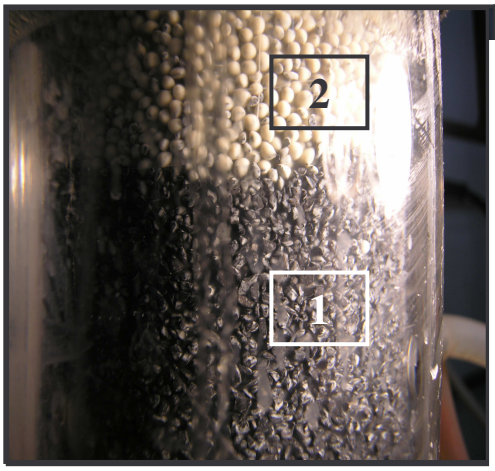

Figure 5 GAC not coated (1) and coated by bacteria during start up of the anaerobic FBBR inoculated with Ecl and Cf1

Figure 6 GAC coated by biofilm with carrier induced granules into the anaerobic FBBR inoculated with Ecl and Cf1 at HRT of 0.5h

With decreasing HRTs the bacterial plate counts for attached bacteria on carrier particles increased from an initial value of 3.0 log cfu/g GAC corresponding to a HRT of 8 h to 8.0 log cfu/g GAC corresponding to HRTs of 1 and 0.5 h. Planktonic bacterial cell counts increased from an initial value of 5.0 log cfu/mL to 8.0 log cfu/mL for HRTs of 1h and 0.5 h, indicating that the high recycle rates compensated for cell loses due to washout at high dilution rates. Bacterial biomass within the bioreactor showed an increase from 31.0±0.0 to 35.7±1.0 g VSS/L in the range of 8-4h HRT, and a decrease to 32.0±0.0 gVSS/L as HRT was reduced from 4 to 0.5 (Table 1).

With continual granule growth the bed expanded to the top of the anaerobic FBBR resulting in washout of granules. A cylindrical filter system placed at the top of the anaerobic FBBR to separate effluent from particulate material became frequently clogged up with granular material and biofilm. Thus it was necessary to clean the

filter on a regular basis. The filter was removed and serviced (cleaned with hot tape H_2O) periodically. The anaerobic FBBR was also replenished with fresh GAC on a regular basis to maintain a standard expanded bed height of GAC at the bottom of the bioreactor.

GAC has been selected as the carrier substrate compared to other carriers (sand, diatomaceous clay, plastic materials) because of its higher surface roughness which offers initial attachment of cells and also protects biofilm cells from shearing. It has also a high adsorptive properties (Zhao *et al*., 1999, Hulshoff Pol *et al*., 2004, Padron, 2004). The use of GAC facilitated formation of a more compact biofilm which resisted to hydrodynamic and liquid flow into the anaerobic FBBR. Additionally, the porous structure of GAC reduced its density and increased the surface area for more bacteria attachment with the advantage of enhancing products formation (Figures 5 & 6). GAC as immobilised carrier material was also successfully used in many other studies (Maloney *et al.,* 2002, Padron, 2004, Lee *et al.,* 2004, Zhang *et al.,* 2007).

3.1.2 Bioreactor monitoring

Different parameters controlled during the course of experiments are presented in Table 1.

All the physical and chemical parameters of the anaerobic FBBR such as temperature, ORP, pH and conductivity remained stable as the HRT was decreased from 8 h to 0.5 h (Table 1). Following the formation of granules and granular bed, the oxido-reduction potential (ORP) and bioreactor conductivity remained constant at -491.3±17 mV and 1293.07±191.4 µS/cm^2 respectively. Temperature and pH were maintained at 37±1°C and pH 5.5±0.3 respectively. As HRTs were decreased below 4 h the subsequent increase of organic loading rates did cause the pH to fluctuate. Fluctuations in pH were minimised by the automated continuous dosing of the recycle stream with either NaOH (6M) or HCl (3M). The large negative ORP values

confirmed the anaerobic and highly reducing status of the bioreactor bed during sucrose degradation. (Ren *et al.,* 2007).

The decision to maintain the temperature and pH constant at the above values was motivated by the successes of previous studies in our laboratory. For example, both Ecl. and Cf1 were successfully grown in the anaerobic FBBR at these temperatures and pH (Thompson, 2005). A pH of 5.5 has been found to be optimal for the anaerobic FBBR process as shown in many other similar studies from the literature (Fan *et al*., 2006, Hawkes *et al*., 2007, Ren *et al,.*2007). Temperature of 37ºC was chosen as the operation was conducted in the mesophilic range (Metcalf & Eddy, 2003).

Table 1 Parameters controlled, VSS production and sucrose conversion (in percentage) obtained from the anaerobic FBBR inoculated with Ecl and Cf1 at different HRTs

HRT (h)	**T (°C)**	**pH**	**ORP (-mV)**	**Conductivity (µS/Cm^2)**	**VSS (g/L)**	**Sucrose conversion (%)**
8	36.8±0.3	5.6±0.1	492.6±15	1291.6±211	31.0±0.0	87.8
6	37.1±0.0	5.5±0.1	490.7±18	1294.9±177	32.7±0.6	87.2
4	37.0±0.1	5.5±0.1	492.1±16	1291.1±183	35.7±0.6	81.8
2	36.9±0.2	5.6±0.1	491.0±17	1292.0±195	32.0±1.0	79.1
1.6	36.8±0.6	5.6±0.2	491.8±16	1285.0±193	31.7±0.6	73.5
1	37.0±0.4	5.6±0.1	490.3±18	1295.3±191	32.0±0.0	72.4
0.5	37.2±0.1	5.6±0.1	490.8±17	1301.6±190	32.0±0.0	72.2

The anaerobic FBBR was operated at least three days at each HRT which allowed sufficient time for the achievement of steady state conditions (Figure 7). Biomass analysis showed an increase from 31.0±0.0 to 35.7±0.6 gVSS/L in the range of 8-4h HRT, then decreased and remained constant at 32.0±0.0 gVSS/L from 4 to 0.5h HRT.

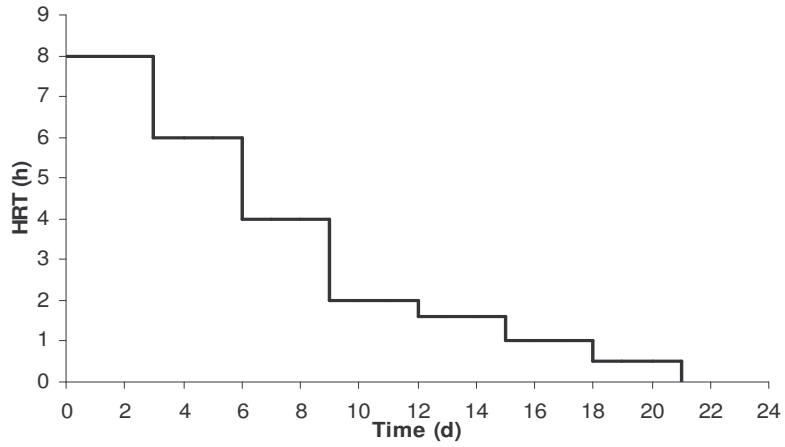

Figure 7 Variation of HRT as function of time during the anaerobic FBBR operation inoculated with Ecl and Cf1

Biomass came from some bacteria attached to GAC which formed biofilm and also from some induced granular sludge particulates which had grown and expanded into the bed. The constant value of VSS from 4 to 0.5h HRT was due to the fact that suspended induced granular sludge particulates were washed out from the anaerobic FBBR at low HRTs. The material lost through washout was continually replaced by the regeneration of fresh carrier induced granular sludge and biofilm attached on GAC. Hence there was always a steady-state or constant bed of microbial biomass for influent substrate transformation and H_2 generation. The excess biomass composed of dead bacteria formed a settled layer or sediment at the bottom of the recycle – reservoir. However, because it remained as undistributed sediment none of this material was recycled back into the anaerobic FBBR. It was removed after the experimental run was completed and the system serviced. Low volumetric biomass production has been known to be one of the advantages of the anaerobic FBBR compared to aerobic processes (Marcoux, 1997, Mahmoud, 2002).

Sucrose conversion decreased with decreasing HRTs (Table 1). At a low HRT of 0.5 h, conversion declined to 72.2% resulting in greater levels of substrate and biomass washout (Lee *et al.,* 2004, Nath *et al.,*2005).

3.1.3 Effect of HRT on H_2 production

Figure 8 represents the influence of HRTs on hydrogen production rates (HPR), specific hydrogen production rates (SHPR) and hydrogen yields (YH_2) of the anaerobic FBBR inoculated with Ecl and Cf1.

Biogas composition for all HRTs with regard to H_2 and CO_2 content remained constant at 42±5.3% (v/v) and 37.6±3.8% (v/v) respectively. CH_4 was below the detection limit of the gas analyser (less than 0.1% (v/v) at the TCD) indicating the absence of methanogenic bacterial contaminant in the anaerobic FBBR. This can be explained by the fact that the anaerobic FBBR was inoculated with pure cultures of facultative anaerobes. pH and HRT were maintained low to stimulate their growth. As a result, any growth of methanogens was inhibited by the above mentioned conditions. A pH value below 8 is not suitable for methanogens. A low HRT is unfavourable for methanogens growth as well; they are known as slow growing microorganisms (Liu & Fang, 2002, Hwang *et al.,* 2004, Leitão *et al*., 2006, Zhang *et al.,* 2007).

HPR was estimated from the measured values for total biogas production rates. SHPR was calculated as the ratio between HPR (expressed in mmol-H_2/(h.L)) and VSS (expressed in g/L). YH_2 was also calculated as a molar ratio between HPR and sucrose consumed. The responses of HPR, SHPR and YH_2 are given in Figure 6. It can be seen that the trend of all graphs were of increase with the decrease in HRTs. This was due to the fact that at low HRT, incoming substrate was directly consumed by immobilised bacteria in form of biofilm and induced granular sludge toward formation of end products (Nath *et al*., 2005, Yang *et al*, 2006, Zhang *et al.,* 2007). A maximum HPR of 138 mmol/(h.L) corresponding to 3.5 L/(h.L) was reached at HRT of 0.5 h. Also, as the HRT was decrease to 0.5 h, the SHPR and YH_2 were 43.12 mmol-H_2/(h.g-VSS) and 4.6 mol-H_2/mol-sucrose respectively.

3.1.4 Effect of organic loading rate on H_2 production

Figure 9 represents the influence of organic loading rate on hydrogen production rate (HPR), specific hydrogen production rate (SHPR) and hydrogen yield (YH_2) of the anaerobic FBBR inoculated with Ecl and Cf1.

The anaerobic FBBR was supplied with a constant influent sucrose concentration of 17.65g/L, thus the organic loading rate (OLR) increased as the HRT was decreased. The influence of increasing OLR on HPR, SHPR and YH_2 is presented in Figure 9.

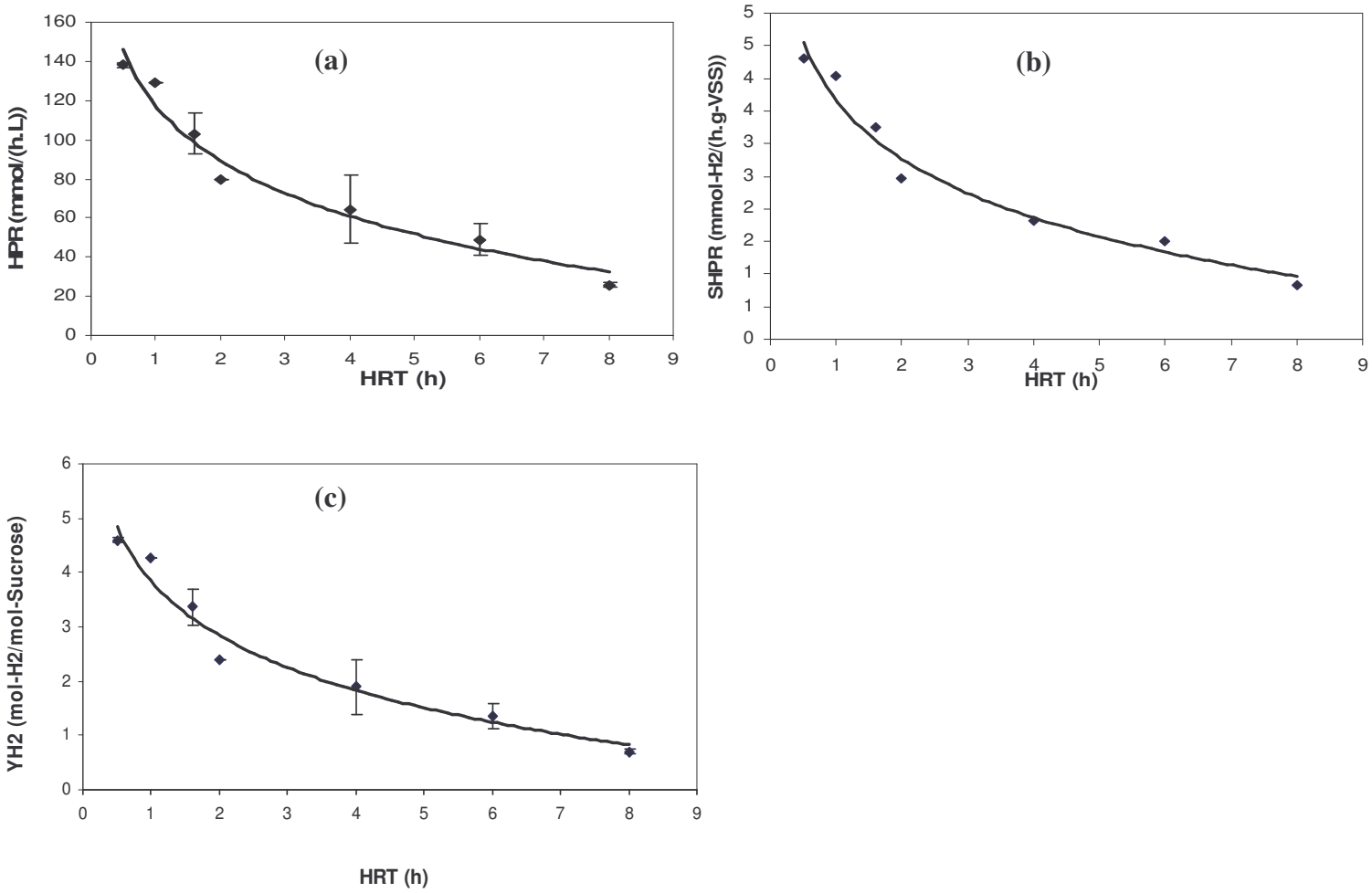

Figure 8 Influence of HRT on hydrogen production rate HPR (a), specific hydrogen production rate SHPR (b) and hydrogen yield YH_2 (c) of the anaerobic FBBR inoculated with Ecl and Cf1 for sucrose degradation

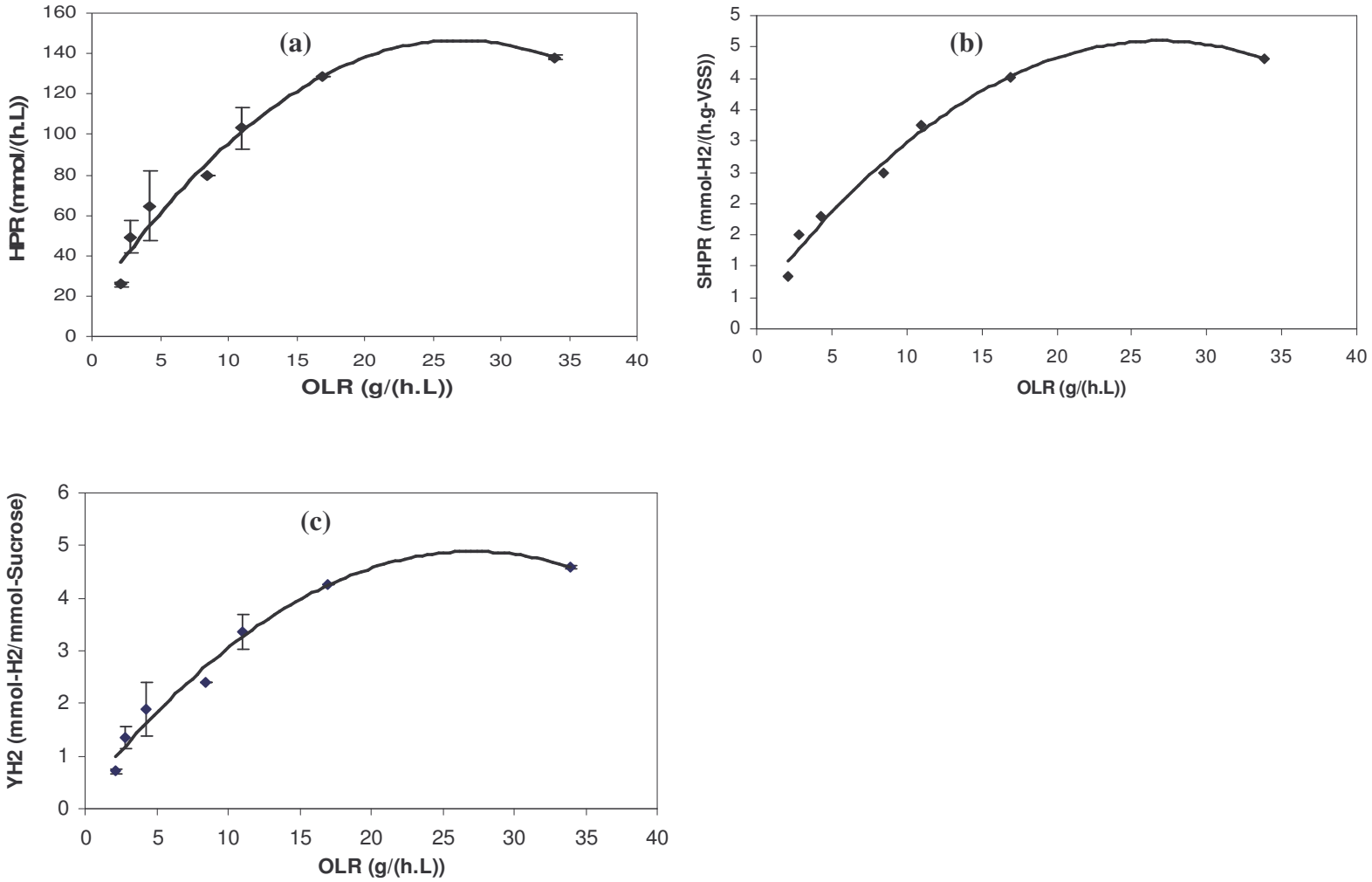

Figure 9 Influence of organic loading rate (OLR) on hydrogen production rate (HPR) (a), specific hydrogen production rate (SHPR) (b), and hydrogen yield (YH_2) (c) of the anaerobic FBBR inoculated with Ecl and Cf1 for sucrose degradation

The hyperbolic shape of curves with respect to increasing OLR rates, resulting from a reduction in the HRT, indicates that the substrate supply for the specific bioreactor volume and for the bioreactor biomass holdup had reached saturating levels. This accounts for the hyperbolic relationship between HPR, SHPR, YH_2 and OLR. When HRT was decreased to below 2 h substrate washout increased (see also Chen *et al.*, 2005). With the OLR approaching 25g/(h.L); HPR, SHPR and YH_2 converged onto their maximum or asymptotic values before decreasing slightly.

3.1.5 Soluble metabolites

Figure 10 presents different VFAs produced during fermentation process in the anaerobic FBBR inoculated with Ecl and Cf1.

Fermentative H_2 production is usually accompanied by production of soluble metabolites (Lin & Chang, 2004b, Steven *et al.,* 2005). Acetic, butyric and propionic acids were the major VFAs present in the anaerobic FBBR, alcohol was not detected. After analysis, it was realised that acetic and butyric acids production were increasing with decrease in HRTs (Figure 10). Acetic acid was in high proportion and varied in the range of 70.6-118.3 mg/L compared to butyric acid (51.1-97.7 mg/L). Propionic acid ranged from 21.8 to 29.8 mg/L and was relatively constant. These values of VFAs concentration were low compared to other studies (Zhang *et al.,* 2007) but they were used to determine the pathway taken by fermentative bacteria during sucrose degradation.

From figure 10, it can be concluded that the anaerobic degradation of sucrose in the anaerobic FBBR was following the acetic acidic pathway and was partially directed to the production of propionic acid. Propionic acid was consuming some of the electrons that would otherwise go into H_2 production. The presence of propionic acid in the anaerobic FBBR effluent could be due to the fact that, as the gas-liquid exchange device was connected to the outlet of the anaerobic FBBR (Appendix 1),

some electrons produced in the bulk solution of the anaerobic FBBR were producing H_2 gas. As H_2 gas was not totally removed, bioreactor microorganisms metabolism were redirecting electrons as well as some H_2 gas to propionic acid production (Hawkes *et al*, 2002, Lin *et al*., 2006).

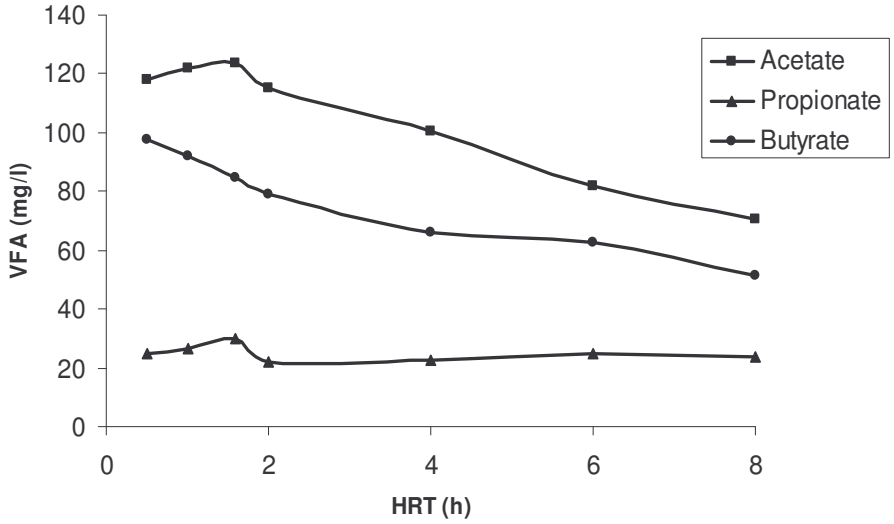

Figure 10 Relationships between VFA (acetate, butyrate and propionate) and HRT in the anaerobic FBBR inoculated with Ecl and Cf1

Theoretically 4 and 8 mol H_2/mol sucrose are yielded when butyrate and acetate are end products in the fermentation process (Lee *et al.,* 2004). This gives a molar ratio of 2:1 which is expected when acetate and butyrate are present in an anaerobic system and that ratio is used as an indicator of the effectiveness of the process for substrate metabolism and H_2 production (Kim *et al*., 2006b).

In this study, the ratio of acetate and butyrate concentrations was randomly varying with HRT. An average of 1.38 proved that the high value of H_2 produced was due to substrate degradation pathway resulted from microorganisms involved in the biochemical reaction.

3.1.6 *Microbial community morphology and characterisation*

Bacterial morphology was determined by scanning electron microscopy (SEM). Rod-shaped cells were observed attached to GAC particles sampled from Ecl and Cf1-containing in the anaerobic FBBR. Ecl and Cf1 are rod-shaped members of the Enterobacteriaceae. Thus, rod-shaped cells, as observed in SEM images, are indicative of their presence (Figure 11) (Liu & Fang, 2002, Liu & Tay, 2004, Wu *et al*., 2005a).

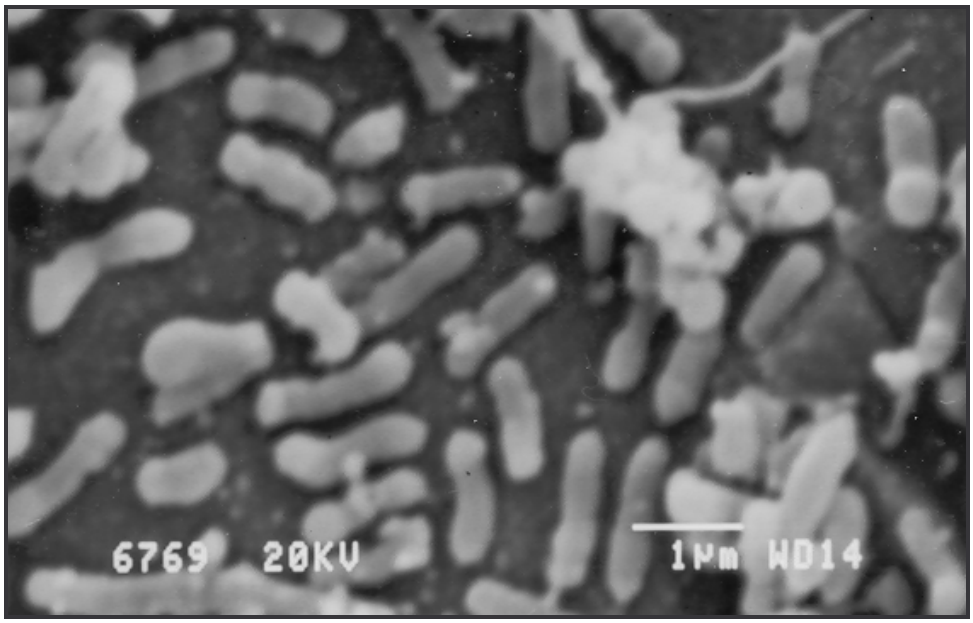

Figure 11 Scanning electron micrographs of consortium of bacteria: Ecl and Cf1 biofilm grown on GAC in the anaerobic FBBR

Single colonies obtained from streak plates on NA of a sample of liquid from the anaerobic FBBR were used for DNA extraction (Ren *et al.*, 2007). Amplification of PCR products showed that size of bacterial DNA was between 200 and 100 base pairs as pictured on figure 12.

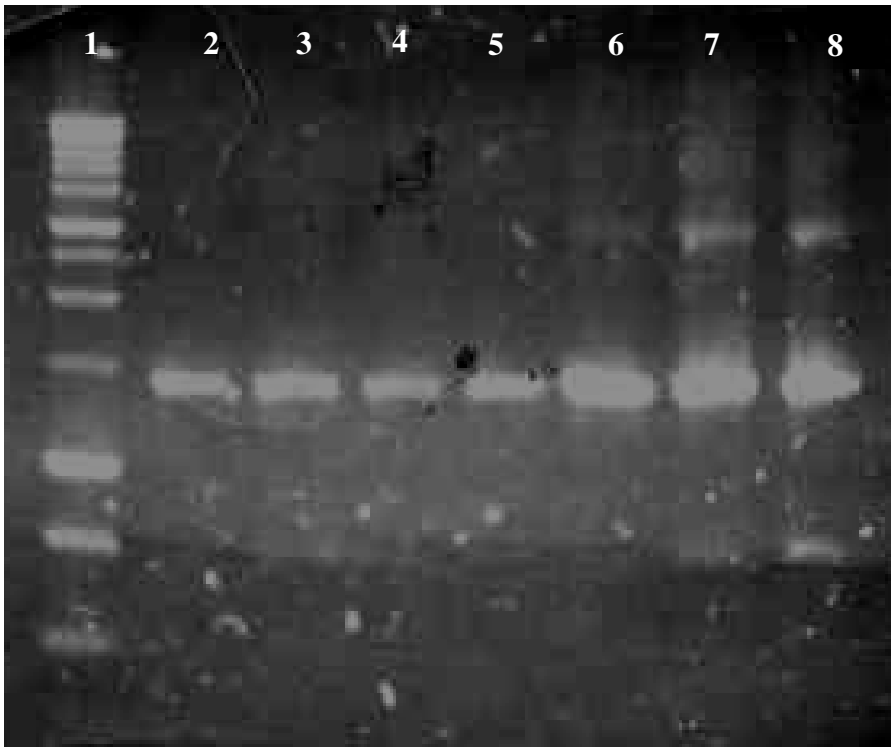

Figure 12 Agarose gel electrophoresis of amplified PCR products coming from bacterial DNA extraction. Lanes 1: Molecular weight maker (Fermentas, Life sciences), Lane 2-8: Bacterial strain samples.

Thereafter, PCR products were sequenced and identified by comparing the 16S rDNA sequences of samples with standard DNA sequences from the Blast server on the NBCI website (Appendix 4.1). Results revealed the presence of these closest species *Enterobacter cloacae* (93%), *Citrobacter freundii* (98-100%) and *Bacillus sp.* (98%) (Appendix 5.1). *Enterobacter cloacae* and *Citrobacter freundii* are facultative anaerobes which appeared in the anaerobic FBBR at different stages of anaerobic treatment. They were responsible of sucrose degradation with production of soluble metabolites and H_2 as end-products (Kumar & Das, 2001, Oh Y. *et al.,* 2003, Kotay & Das, 2007). *Bacillus species* appeared in the anaerobic FBBR as contaminants from the environment since the bioreactor was operated as an open system. Fortunately, some species of this genus are known to degrade organic materials with production of H_2. This fact contributed to high H_2 production rates in the present study (Shin *et al.*, 2004).

3.2 Performance of the anaerobic FBBR for H_2 production using undefined mixed cultures of bacteria

In this experiment a mixed culture of bacteria from activated sewage sludge was used as inoculum in the system. All system parameters such as temperature and pH were maintained constant. Prior to bioreactor operation, the inoculum was pre-treated as described in chapter 2 in order to enhance its H_2 productivity and to kill possible pathogens (Zurawski *et al.,* 2005). The anaerobic FBBR was operated with a constant substrate concentration at different HRTs; thereafter the effect of influent substrate (sucrose) concentration on H_2 gas production was also studied.

3.2.1 *Anaerobic FBBR start up*

Figure 13 shows the anaerobic FBBR at steady state operation with GAC coated by mixed undefined bacteria which formed biofilm.

The anaerobic FBBR was first run with a constant influent sucrose concentration of 17.65 g/L (20 gCOD/L) as performed in the previous experiment in order to get biofilm attached to GAC. The recycle flowrate was maintained at 1.5L/min. Start up took approximately 25 days when the HRT was sequentially reduced from 8 to 4 h. This long start up (compared to the previous experiment) was due to acclimation of the inoculum which acquired a relative extended period of time to get attached to GAC. Growth of biofilm and formation of granules occurred in the anaerobic FBBR and bed expansion occurred with granule production (Figure 13). The OLR was increased during start up from 2.12 to 4.24 g/(h.L). Thereafter, planktonic and attached bacterial counts were measured around 2 log cfu/mL and 8 log cfu/g-GAC respectively.

Figure 13 GAC coated with mixed undefined bacteria biofilm at 0.5 h HRT during the anaerobic FBBR operation

After start up, when the anaerobic FBBR bed was expanded to 100% of the anaerobic FBBR volume, HRT was reset to 8 h and then it was gradually decreased until it reached 0.5 h HRT. At the same time, control of parameters (temperature and pH), monitoring of sucrose consumption and products formation from the bioreactor were performed. Biogas analysis showed an H_2 content of approximately 42 % (v/v) which was used to estimate the HPR.

3.2.2 *Anaerobic FBBR operation*

Table 2 depicts the status of the anaerobic FBBR inoculated with a mixed undefined culture of bacteria from sewage sludge at different HRTs.

Sucrose degradation increased from 65.3% at HRT of 0.5 h to 88.3% at HRT of 8 h. This increase was inversely related to HPR which varied from 20.9 mmol/(h.L) at HRT of 8 h to a maximum of 130.1 mmol/(h.L) at HRT of 0.5 h (Table 2). YH_2 followed the same trend as HPR achieving a value of 4.8 mmol-H_2/mmol-sucrose at

Table 2 Status of the anaerobic FBBR inoculated with a mixed undefined culture of bacteria at different HRT

HRT (h)	T (ºC)	pH	ORP (mV)	Conductivity (µS/cm²)	HPR (mmol/(h.L))	OLR (g/(h.L))	Sucrose conversion (%)
8	37.0±0.1	5.6±0.1	450.3±8	1239.5±104	20.85	2.12	88.3
6	37.1±0.2	5.6±0.0	450.5±8	1221.9±102	47.40	2.82	86.1
4	36.9±0.3	5.6±0.0	451.0±8	1237.04±93	60.07	4.24	77.6
2	36.9±0.2	5.6±0.0	451.0±7	1247.67±92	92.45	8.47	72.9
1.6	37.1±0.1	5.5±0.0	450.5±8	1252.81±85	99.13	11.03	71.4
1	37.0±0.2	5.6±0.0	450.8±8	1231.9±104	123.96	16.94	66.5
0.5	37.0±0.1	5.6±0.0	450.4±8	1243.6±101	130.13	33.89	65.3

HRT of 0.5 h as depicted in Figure 14. The decrease in sucrose conversion was due substrate washout corresponding to low HRT.

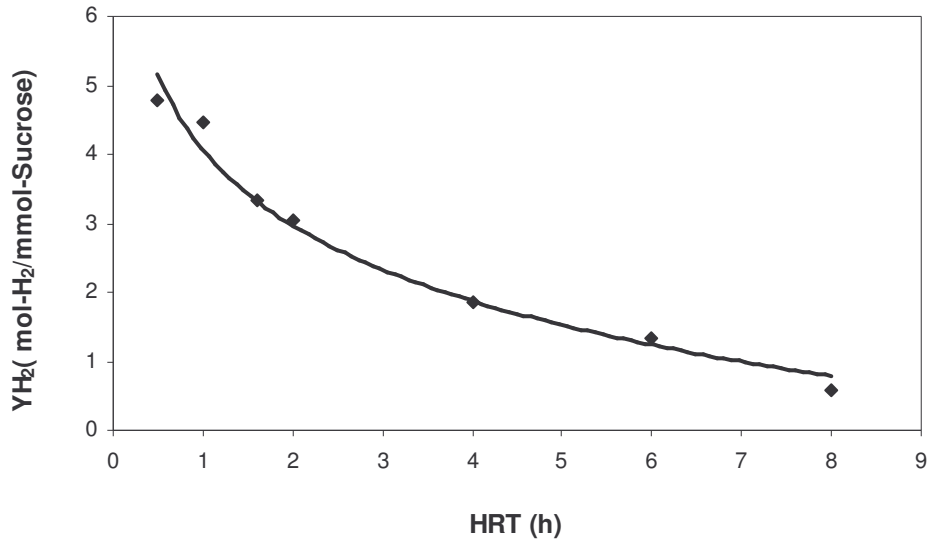

Figure 14 Relationships between YH_2 and HRT for the anaerobic FBBR inoculated with undefined mixed culture of bacteria

By observing the figures above (Table 2 and Figure14), HRT of 2 h seemed to give the OLR for optimal sucrose conversion and the maximum values for HPR and YH_2.

This could be due to increasing growth of H_2 producing microorganisms in the bioreactor which enhanced sucrose degradation (Sung *et al.,* 2003). The performance of the anaerobic FBBR was then evaluated in terms of influent sucrose concentration which was varied in the range of 10-40 gCOD/L at a fixed HRT (2 h).

3.2.3 *Influence of sucrose concentration on HPR*

As shown in figure 15, sucrose conversion increased with the influent sucrose concentration in the range of 10 – 25 gCOD/L, then it declined with a polynomial trend reaching a value of 41.4 % at an influent concentration of 40 gCOD/L.

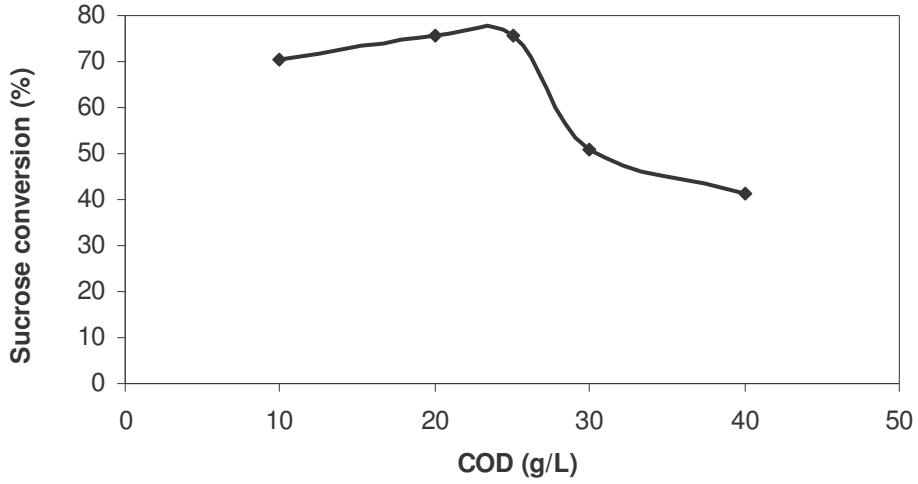

Figure 15 Sucrose conversion as function of influent sucrose concentration in the anaerobic FBBR inoculated with undefined mixed culture of bacteria

The trend of the curve can be explained by the fact that bacterial degradation of sucrose is accompanied with biomass formation. The increase in biomass is related to the time that biochemical reactions are lasting in the anaerobic FBBR and they consume some of the substrate (sucrose) feed to the bioreactor. Sucrose is also partly directed to VFAs production, especially butyrate, which consume some of the substrate as reported by Liu and Fang (2002). It can be seen from figure 15 that 20

gCOD/L was the optimal sucrose concentration. This explained why this concentration has been used in many studies (Chang *et al.,* 2002, Lee *et al.,* 2004, Chen *et al.*, 2005).

The HPR was calculated considering an H_2 content of 42% (v/v) (result not shown). It was increasing linearly with the increase of influent sucrose concentration (Table 3) and attained a value of 88.51 mmol/(h.L) at sucrose concentration of 40gCOD/L. At 20 gCOD/L of sucrose concentration, HPR was 50.58 mmol/(h.L) which was lower compared to the one presented in Table 2 (92.45 mmol/hxL) for biofilm and granule formations. This was due to OLR shock related to influent sucrose concentration which reportedly has a direct effect on substrate degradation (Lietão *et al*., 2005).

Table 3 Operational conditions, HPR and VFAs produced from the Anaerobic FBBR inoculated with undefined mixed culture of bacteria at different influent sucrose concentration and at fixed HRT

COD (g/L)	**HRT (h)**	**T (°C)**	**pH**	**HPR (mmol/(h.L))**	**Acetate (mg/L)**	**Butyrate (mg/L)**	**A/B**
10	2	36.3±0.3	5.6±0.1	26.50	110.75	168.84	0.66
20	2	36.1±0.1	5.6±0.1	50.58	58.48	130.86	0.45
25	2	36.4±0.3	5.6±0.1	56.20	70.07	136.54	0.51
30	2	36.3±0.4	5.6±0.1	70.81	104.59	144.77	0.72
40	2	36.2±0.1	5.6±0.1	88.51	108.73	162.64	0.67

YH_2 decreased first until it reached an optimum of 1.61 mmol-H_2/mmol-sucrose at 20 gCOD/L then increased with a polynomial trend attaining a value of 2.52 mmol-H_2/mmol-sucrose at a concentration of 40 gCOD/L (Figure 16). This tendency is due to sucrose consumption which was high at influent substrate concentration of 20 gCOD/L.

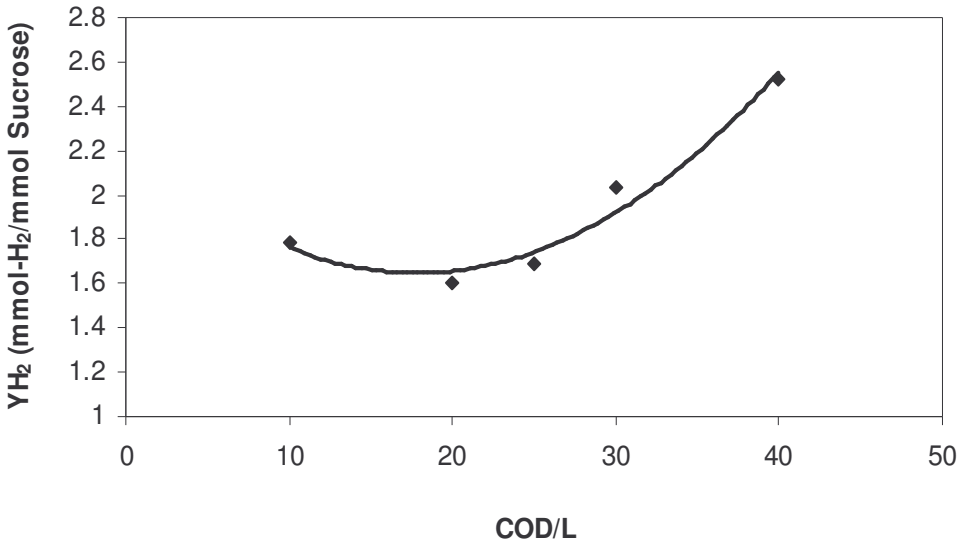

Figure 16 YH_2 as function of influent sucrose concentration in the anaerobic FBBR inoculated with mixed undefined culture of bacteria

3.2.4 *Volatile fatty acids*

An analysis of the liquid bioreactor revealed the presence of acetate (58.48-110.75 mg/L) and butyrate (130.86-168.84 mg/L). Propionic acid was below the detection limit. The process was following the butyric acid pathway as the acetic and butyric acids ratio (A/B), which is used to estimate the pathway was varying from 0.45 to 0.72 (Table 3) (Lin *et al*., 2006). This explained the low substrate conversion as reported by Liu and Fang (2002).

3.2.5 *Bacteria morphology and characterisation*

To determine the morphology of the attached microorganisms, SEM was performed from samples of bacterial biofilm. An image of biofilm microstructure attached on GAC showed some rod shaped colonies which seemingly could be attributed to the presence of acidogenic bacteria (Figure 17), but any sustained conclusion could not be taken as SEM is a qualitative tool as started in the previous experiment.

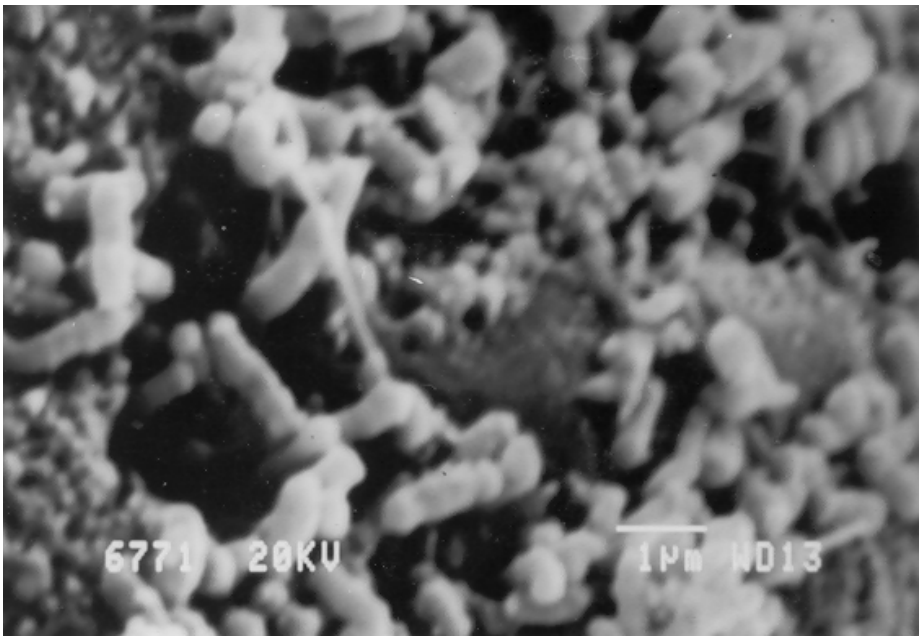

Figure 17 Scanning electron micrographs of consortia of mixed undefined bacterial biofilm grown on GAC

Therefore DNA extraction was performed on single colonies isolated from bacterial biofilm samples (Ren *et al.*, 2007). They were amplified and then run on 1% agarose gel. An image of the agarose gel with amplified PCR products is shown on figure 18.

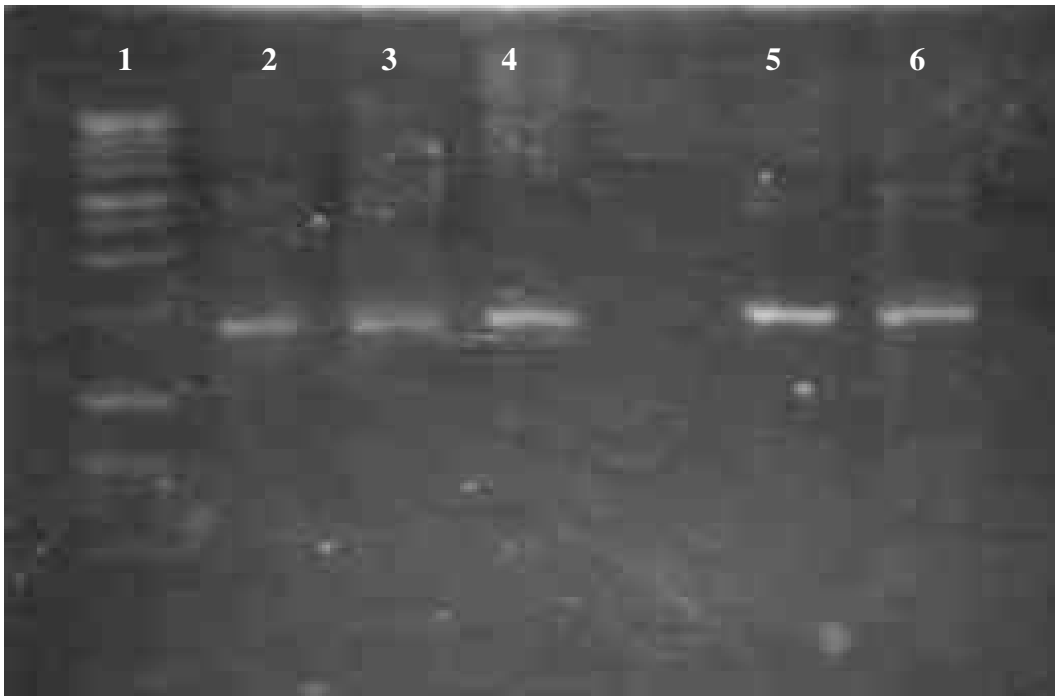

Figure 18 Agarose gel electrophoresis of amplified PCR products coming from bacteria DNA extraction. Lanes 1: Molecular weight maker (Fermentas, Life sciences), Lane 2-6: Bacterial strains.

As it can be seen from the agarose gel picture, the amplified PCR products corresponded to a size between 200 and 100 base pairs which confirmed the presence of bacterial strains.

By comparison of the 16S rDNA sequences with standard DNA sequences as explained in chapter 2, results showed that many colonies were uncultured (Appendix 6.2). The closest colonies identified such as *Prevotella enoeca, Flavobacterium and Bacteriodetes symbiont* were matching partially at 84%, 83% and 83% respectively (Appendix 5.2). They confirmed the presence of rod shaped colonies observed from SEM image and they are recognised as metabolically capable for breaking down proteins and carbohydrates with production of VFAs and H_2 gas production (Shin *et al.*, 2004, Ren *et al.*, 2007, Yang *et al.*, 2007).

3.3 Comparison with other studies

Table 4 Comparison of the anaerobic FBBR performance with other studies

Microorganisms	Bioreactor Type	Substrate Concentration (g/L)	Hydrogen production rate (L/(h.L))	References
Sewage sludge	CSABR[a]	Sucrose (26.7)	14.5	(Wu *et al.,* 2005b)
Sewage sludge	CIGSB[b]	Sucrose (17.8)	7.3	(Lee *et al.,* 2004)
Sewage sludge	DTFBR[c]	Sucrose (17.8)	2.27	(Lin *et al.,* 2006)
Ecl IIT-BT 08	PB[d]	Glucose (10)	1.85	(Kumar & Das, 2001)
Sewage sludge	FBBR	Sucrose (17.65)	3.308	This study
Ecl and Cf1	FBBR	Sucrose (17.65)	3.508	This study

[a] CSABR: Continuous stirred anaerobic bioreactor

[b] CIGSB: Carrier induced granular sludge beds

[c] DTFBR: Draft tube fluidized bed reactor

[d] PB: Packed bed

[e] FBBR: Fluidised bed bioreactor

In Table 4, different bioreactor systems have been chosen from the literature to compare their H_2 production rates with results obtained in this study. All bioreactors were working anaerobically using either sucrose or glucose as carbon source. They were inoculated with known bacterial strains or microorganisms from activated sewage sludge which were attaching to suspended solid materials used as bioreactor beds.

In all systems, HRTs were reduced gradually in order to increase nutrient availability to microorganisms and in some bioreactors such as CSABR, DTFBR and also in the anaerobic FBBR used in this study, values as lower as 0.5H HRTs were achieved. Consequently, bacteria grew in the bioreactors in form of biofilm giving rise to granules which increased sucrose or glucose degradation.

Results from our experiments showed that the performance of the anaerobic FBBR used for biohydrogen production when compared to other studies, was quite interesting as maximum values of HPR (3.51 L/(h.L)) and HPR (3.31L/(h.L)) were achieved using Ecl and Cf1 strains and also mixed culture of bacteria from an activated sewage sludge respectively. High rates of biomass retained into the bioreactors and their configurations could explain higher HPR obtained by Wu *et al.* (2005b) and Lee *et al.* (2004).

CHAPTER FOUR - SUMMARY AND CONCLUSIONS

The present study focused on the production of H_2 using a synthetic wastewater that had sucrose as the carbon source in an anaerobic FBBR.

An anaerobic FBBR have been chosen to perform this study because of many advantages (when compared to other type of bioreactors) such as higher biomass concentration, lower hydraulic retention times, higher volumetric removal rates and relatively small area requirements. These properties allowed getting growth of bacteria granules and fulfilling objectives assigned to this study.

In the first experiment two facultative bacteria Ecl and Cf1 were used as inoculum in the anaerobic FBBR packed with GAC as carrier material. They were identified, characterised and submitted in GenBank under the following accession numbers: Cf1 (EU046372) and Ecl (EU046373). According to operating conditions, these isolates were able to attach and grow to the carrier material giving rise to biofilm. Attachment of bacteria to solids could be due to particle charges and to the production of extracellular polymeric substances (EPS) at some stage of their growth which are considered as source of biosorption (Mahmoud *et al.*, 2003).

It seemed that biofilm was a necessary step in the carrier induced granule formation process. Biofilm and induced granules play an important role as biocatalyst in the degradation of carbohydrates contained in wastewater. Granule formation was found to be very sensitive to HRT and was only initiated when linear flow rates became greater that 1 L/min. This corresponded to a certain degree of bed porosity and avoided any friction between particles which in some case conducted to bacteria detachment from the carrier material.

GAC used in the anaerobic FBBR was heterogeneous with respect of particle size (0.6 to 1.1 mm) and shape; however it appeared to be sufficiently suitable for biofilm

production and granule formation. This was due to its adsorptive properties and also to its irregular external surface.

The presence of biofilm and granular sludge increased bacteria biomass holdup in the anaerobic FBBR, which resulted in high specific bioreactor reaction rates (sucrose degradation). For example, as HRT was reduced the fluidised granular bed biomass density increased rapidly and at the same time the HPR also increased to its maximum of 138 mmol H_2 /(h.L) at HRT of 0.5h. This trend was expected as the reduction of HRT is related to the increase of organic loading rates.

The values of volatile fatty acids were low when compared with other studies. We did consider the obtained values only as an indication of substrate degradation pathway. A differential mass balance was performed on the influent substrate and effluent products formation from the anaerobic FBBR; a huge difference has been observed between the two data. Any sustained conclusion could not have been taken on the obtained results.

In the second study, the anaerobic FBBR was inoculated with undefined mixed culture of bacteria to study the influence of HRTs on HPR. As for the first experiment, the HRT was decreased gradually and the HPR also underwent a corresponding increase. A maximum of 130.1 mmol H_2/(h.L) was reached for a HRT of 0.5h. This demonstrated that from a practical point of view, undefined mixed culture of bacteria could also be used for H_2 production; more importantly, a pre-treatment will be needed to kill possible microbial pathogens and methanogens. It is essential to remove all methanogens in order to achieve enhanced level of H_2 production.

This study also confirmed that the best influent sucrose concentration at HRT of 2h was 20gCOD/L . This gave the highest sucrose conversion similar to other studies on biohydrogen production using sucrose as the carbon substrate.

This study is a contribution to research on source of energy which could replace energy of fossil fuel origin and provided procedures for optimising biological production of H_2 gas as an environmental friendly source of energy. The H_2 gas produced could be used to power a fuel cell for electricity generation.

A limitation in this study was the investigation on the effects of microbial diversity in the FBBR on substrate degradation pathway. Also future studies should focus on the population and community dynamics of the mixed species consortium in the granular bed of the anaerobic FBBR. This will require the identification of the bacteria species in the communities and how their frequency affects the biochemical pathways for substrate metabolism and biogas production.

Further studies should investigate the influence of temperature on the bacterial community in order to ascertain whether they can adapt to thermophilic temperature regimes in the bioreactor. Thermophilic temperatures offer the benefit of pathogen free bioreactors and enhanced HPR levels. In addition the influence of a more heterogeneous and complex carbon substrate that includes cellulosic materials for H_2 gas production should be the focus of further studies.

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APPENDICES

APPENDIX 1

Photograph of the fully automated set up used for biohydrogen production from synthetic wastewater

APPENDIX 2

Source of chemicals, equipments and suppliers

Chemical and equipment	Supplier
Agarose	Saarchem
Autoclude Peristaltic Pump	Wirsam Scientific
Boyser AMP-16 Peristaltic Pump	Aquapump
C_2H_5OH, K_2HPO_4, $MgCl_2.6H_2O$, $FeSO_4.7H_2O$, $CoCl_2.H_2O$, Nutrient agar	Merk (South Africa)
Data Taker DataLogger	Measurement & Controlsystem
Electrode housing vessel	A-Z Technical services
Ethidium bromide	Saarchem
Feed Bins, Reservoir	Perspex World
Grant Digital 60 Waterbath	Laboratory Automation and Control
HCl (32%), Granular activated carbon	Associated Chemical Enterprises
$MnSO_4.4H_2O$, $CuSO_4.5H_2O$, KCl	BDH (Merk, South Africa)
NH_4HCO_3, $NaHCO_3$, NaOH flakes	Protea Industrial Chemicals (South Africa)
Nutrient agar	Biolab

Chemical and equipment	Supplier
Nutrient broth	Biolab
Perspex for reactor construction	Mazey's Plastics
pH, Conductivity, Redox probes, Thermocouple	Swiss lab
Plumbing components	Leeways Garden Centre
Resorcinol	Fluka
Silicon grease	Evna Industrial Products
Sucrose	Pick and Pay (South Africa)
Wiring, Resistors, Control System Housing	AP Electronics

APPENDIX 3

Media composition

Media formulation with C:N:P ratio of 334:42:1 (Endo *et al.,* 1982)

Chemical Component	g/L
Sucrose	17.8
NH_4HCO_3	5.24
K_2HPO_4	0.125
$NaHCO_3$	6.72
$MgCl_2.6H_2O$	0.1
$MnSO_4.4H_2O$	0.015
$FeSO_4.7H_2O$	0.025
$CuSO_4.5H_2O$	0.005
$CoCl_2.H_2O$	1.24×10^{-4}

Modified Endo formulation with a C:N:P ratio of 334:28:5.6 (Thompson *et al.,* 2006)

Chemical Component	g/L
Sucrose	17.65
NH_4HCO_3	3.49
K_2HPO_4	0.699
$NaHCO_3$	6.72
$MgCl_2.6H_2O$	0.1
$MnSO_4.4H_2O$	0.015
$FeSO_4.7H_2O$	0.025
$CuSO_4.5H_2O$	0.005
$CoCl_2.H_2O$	1.24×10^{-4}

APPENDIX 4

Isolation of genomic DNA from bacteria

(Method from InstaGene matrix, catalogue # 732-6030)

1) Pick an isolated bacterial colony and resuspend it in 1 ml of autoclaved water in a microfuge tube.
2) Centrifuge for 1 minute at 10,000 – 12,000 rpm. Remove the supernatant.
3) Add 200µl of InstaGene matrix to the pellet and incubate at 56ºC for 15-30 minutes. **Note**: InstaGene matrix mix should be mixed at moderate speed on a magnetic stirrer to maintain the matrix in suspension. The pipette tip to be used should have a large bore, such as a 1,000µl pipette tip (Bio-Rad's catalogue # 223-9378).
4) Vortex at high speed for 10 seconds. Spin at 10,000-12,000 rpm for 2-3 minutes.
5) Use 20µl of the resulting supernatant per 50µl PCR reaction. Store the remainder of the supernatant at -20ºC. Repeat step 5 when reusing the InstaGene DNA preparation.

APPENDIX 5

Gel electrophoresis

Agarose Gel (50ml)

Agarose (0.5g for 1%)
10ml 5X TBE
40ml distilled water
Heat until agarose has completely dissolved
Add 1 µl Ethidium Bromide

5X TBE

54g Tris base
27.5g Boric acid
20ml 0.5M EDTA pH 8.0
Make up to 1L with distilled water and autoclave at 121ºC at 15psi for 20 minutes

Electrophoresis buffer

100ml 5X TBE
900ml sterile distilled water
2.5µl Ethidium Bromide

APPENDIX 6

Appendix 6.1 Sequences of single colonies extracted from the anaerobic FBBR inoculated with *Enterobacter cloacae* and *Citrobacter freundii*.

Sequence of sample 2:

AATGTGTACACAGCGCGCCCGCGTATATAAACATGCAACTTGAAGGTAGC
ACAGAGGAGCTTGCTCCTTGGGTGACGAGTGGCGGACGGGTGAGTAATGT
CTGGGAAACTGCCCGATGGAGGGGGATAACTACTGGAAACGGTAGCTAA
TACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCC
ATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACC
TAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAAC
TGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA
CAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTT
CGGGTTGTAAAGTACTTTCAGCGAGGAGGAAGGCGTTGTGGTTAATAACC
GCAACGATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAG
CAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGT
AAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCA
ACCTGGGAACTGCATCCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGG
GTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATAC
CGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGTCGACTTG

Sequence of sample 3:

TCGGGACGCCGCGCGGCGCGTAATAAACATGCAAGTCGAAGGTAGCACAGAAG
AAGCTTGCTCCTTGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGAAAACT
GCCCGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCG
CAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGG
GATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCT
GAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAG
GCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCTTGCCGCG
TGTATGAAGAAGGCCTTCGGGTTGAAAAGAACTTTCAGCGAGGAGGAAGGCGTT
GAGGTTAATAACCGCAACGATTGACGTTACTCGCAGAAGAAGCACCGGTTAACT
CCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTG
GGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTC
AACCTGGGAACTGCATCCGAAACTGGCAGGCTAGAGTCTTGAAAAGGGGGGTAG
AATTCCAGGTGTAGCGTGTGAAATGCGTAAAGATCTGGAGGAATACCCGGTGGC
GAAGGCGGCCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGCCAACTCC

Sequence of sample 4:

GACGACAGCTGGCGGCAGACACATAAACACATGCAGTCGAACAGGTGGC
ACAGAAAGCTTGCTCTCGGGTGACGAGTGGCGGAACGGGTGAATTATAG
CCTGGGAAAACTGCCTGATGGAGGGGGTAAAACTACTGGGAAACGGTAG
CTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCT
TGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGGGGGGTAACGGCT
CACCTAGGCGACGATCCCTAGCTGGCCTGAGAGGATGACCAGCCACACTG
GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGGGGGGAATAT
TGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATAGAAGAA
GGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGGGGTTA
ATAACCGCAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCG
TGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCCGATATTAC
TGGGCGTAAAGCGCACGCAGGCGGCCTGCCAAGTCGGATGTGAAATCCC
CGGGCTCAACCTGGGAACTGCTTTCGAAACTGGCAGGTTAGAGCCTTGTA
AAGGGGGGCAAATTTCCAGGTGTACCCGTCGAAATGCGTAGAGACCCGG
AGGAACCCGGTGGCGAAGGCGGCCCCCTGGAAAAAGACTCGACGCTCAG
GTGCGAATGTTGGGGAGCCAACGGGTTTAAATACCTGTGTATCCCCCCTC
GTTTACTATGTCCATAG

Sequence of sample 5:

GTATAGCAGTGCAGGGTGGCATTCTGAAAACTTTACTAGCGGGGCCTCAC
TTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACATACTTTATG
AGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATATGCCATTGTAGC
ACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCA
CCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACCG
CTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACAT
TTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCC
GAAGGCACCAAAGCATCTCTGCTAAGTTCTCTGGATGTCAAGAGTAGGTA
AGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCGG
GCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCG
GTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTC
CAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTT
GCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCT
TCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGA
ATTCTACCCCCCTCTACAAGACTCTAGCCTGCCAGTTTCGGATGCAGTTCC
CAGGTTGAGCCCGGGATT

Sequence of sample 6:

CGGCTTAGCATGTGCGACGGATGCGCATGTCGTGATGCGCTAGTTGACTA
GCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGA
CATACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATAT
GCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGA
CGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCC
CGGCCGGACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGAC
TTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGT
CTCAGAGTTCCCGAAGGCACCAAAGCATCTCTGCTAAGTTCTCTGGATGT
CAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCC
ACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCG
TACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCA
AGGGCACAACCTCCAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTA
TCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTC
CAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTCAC
CGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCTAGCCTGCCAGTTT
CGGATGCAGTTCCCAGGTT

Sequence of sample 7:

GTAGTAGTAGTGGATTGGACATGCGTGATGCACGCATTACTAGCGATTCC
GGCTTCATGGGAGCGAGTTGCAGCCTCCAATCCGAACTGGGAATGATTTT
ATGGGATTGGCTCCCCCTCGCGGGTTGGCAACCCTCTGTATCATCCATTGT
AGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCC
CCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTAAA
TGGCTGGGAAACTAAAATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCA
ACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACCACTG
TCCCCGAAGGGAAAGATGTATCTCTACACCGGTCAGTGGGATGTCAAGAC
CTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTT
GTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCC
CAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCC
CCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAA
TCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGACCAGAA
AGCCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTA
CACGTGGAATTCCGCTTTCCTCTTCTGTACTCAAGTCCTCCAGTTTCCAAT
GACCCTCCACGGTTGAG

Sequence of sample 8:

GTGCATGTATGTCAGCGCGTAGCATGCTGATCTACGTATTACTAGCGATTC
CGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACTT
TATGAGGTCCGCTTGCTCTCGCGAGTTCGCTTCTCTTTGTATGCGCCATTG
TAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATC
CCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGG
ACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCA
ACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGAGT
TCCCGAAGGCACCAATGCATCTCTGCTAAGTTCTCTGGATGTCAAGAGTA
GGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGT
GCGGGCCCCCGTCAATTCTTTGAGTTTAACCTTGCGGCCGCACTCCCCAGG
CCCTCACTTAACGCGTTAGCTCCGGAAGCCTCTCCTCAAGGCAC

Appendix 6.2 Sequence of single colonies isolated from the anaerobic FBBR inoculated with mixed culture of bacteria from activated sewage sludge

Sequence of sample 2:

GTACCACGGTATCCAAGTATTTTATCCACATGCATAAAACCGGCTTCCCTC
CCAGCGGGGTCGCTGCCTCTGCGACGTCTCCTCGAGTTGGGCAAGATTCC
CCACTAGTTGACCCACATACGCACACTGGAGCGTGCCCTCAGGAGTTTGG
AGCGCGACCCCCCTCCAAGGTGGGGTACCCTCCTCTCCCATGCCGATGCT
GTTCCCTCGCCTGGTGGGTACAGTGCCCCCGCCAACTGTCTAATAACACA
GCATCTCCCTTTAACATACTGATGAATGTGAAGCTCGCACATTAGATGTG
CGTGCCGAGGAGGCACATAAGGAATGGCATAGTCCGCTCTTTCAAGCAGG
CTAGTCCACTTAGTGTGTCGAGCAACGTTGGACTAGCCGTTACTCACCCGT
GCGCCGGTCGCTATCCTTGGTTGCAAAACAACCTAGATGACGCCCCTCGA
CTTGCATGTGTTAGGCCTGGGCCCCCCGTGCCCCCGCCCCGCCCGCCGCGT
GCAACGGAAGAATTCGTTTTATGTGGTTAAGGACCACTTTCTTTGCACTGG
TTTTGTATGCTTTTGTGGGTACGGACACCCACCCCCCCGCGCTTGATCCCT
ATTCTTCTTCTCTTGTTTCTGCGTGTGGATTCTTCGTCTGGCAGCTGGTGCG
ACACCCGGGTAGGGATCTCTTCAGTGTGTCCGGCTTCGCCCTGTTCTTCAT
CGTCTCTCTGATATTGACATCGTGTCTGCTCTGACTGCGTGCACGCGCGGT
GTGCTGCCGCCGCTCTCTCGTGCTTCGCAGCACCGTACACGCCACCGCGC
ACCTTGCTCGCGCATGCTGTGTGTCCCTGTTCCTGTCTCG

Sequence of sample 3:

TCACCGTGCTTGATCGTACACGAACCATGGATCTTTACCACGCAGAAGAC
CGGCATGACTCCCGCAGGGTCTAGCTGCCTCGGGACTTTCGCCGGTTGGG
ACAATAGTTCCCACCTGTTGACTCACATACGAGACTGGAGCGTGCCTCAG
GACCATAGGAGCGCGATCACCTTCTAATGTGGGCTACCCATCCTCTGACT
ATGGCGAGCCGTTACCTCAGCCAAGGTGGCTACATGCCCCGCGGCGAACC
AGTCCTAATAGCGAGATTATCCCCTTCAATTCACCCGATGAATGCGAAGC
TCGCACATATTAATGTGTAATTAAGGCAGCGCACATTATAGGGATGGCTA
GTCCCGCCTTCTCAAGCAGGTTAGTTCACTTACGTGTTGGAACAGCTGGA
CACCCGTTAGATCACCCGAGCGCCGGTCGCTCATACTCGGATGCAGAGAC
AGCCGGGGTGACGCCCCTCGACTTGCATGCGTTAGGCCTGGGCCCCCCGT
GCCCCCGCCTCGTCCGCTGTGTTTTATGTTAGATTTTTTTAAAAAAAAAAT
TAATTCTGTCATTAGGATCGCCGATGTGCTTTTGTTGTTGTGGTCTCGTCG
CATGCGGCACCCTCGCCTCGGTCCACGTGTTTGAGTACACGCGCGTGCTC
ACTCTTCATGCAGCGCTGTCTCGGGCCAGGCGGGCGCCACGCCCGCTATA
TCCCTTGTACCACAACACTGCGAGCACGACGCGCGGGTTGAGCGCACGGC
CGCGTTGCTGGGATGCTGTGCTGCGAGTGCGCGATCTCGCGCCAGCGCAC
TGCCCCTGCGTCCGTCTCGTCAGCGTCACTGCAACAGCCACTGTCGCGAG
AAGCGCGACTCA

Sequence of sample 4:

GTACGTGTCTCATAGTGGTACACGTCCCGCGATTTATCCCCGCATAAAAG
CAGTTTACACTCCCGTGGGGCCGTCGTCTCTGCACGCTCACTTGGCTGGGC
CAGGCTCTCAGCCCAGCTTGACCAATATACCACACTGGAGCCTCCCGTAG
GAGTTGGGACCGCGACTCACTTCCAATGTGGGGTACCTTACTCTCCTAAC
CCGCTACTGTATCGCCGGTTTGGTGGGCCGTGACCCCGCCAACTGCCTAA
TCAGACGCATCCCCATCACATACCGATGAATGCTTTACTCCACATTAGAT
GACTGCCGTGGAGGACATAAGGAATGTATGGTCCGTCTTTCAACGGGTTA
TCCCTTAGTGTGCGGAAGGTTGGATACCCGTTACTCACCCGTGCGCCGGT
CGCCATCATCGTTTGCAAGCGAACCGACATGATGCCCCTCGACTTGCATG
TGTTAGGCCTGGGCCCGCCGTGCCCCCGCCCCGCCCGCCGCGCGCGGGGG
GTGATTTATTAATAAAAATCACTATTTTTGCTTTTGTTTTTCTCACTACGAT
CGTGGCTCCCTTTGCGGACAGCCTTCGTCTCTCTGTGTGTGTGTCTCTGCC
GGCGCGTGGGCGGCGGGCGGCGCGATCGCATAACCATCTCTTCCGAAGG
GAGCCCTAGCCGGCGACAACCAGCCCTCCTCGATCCTCCACCCGGCTGCG
CTGGCGTGTCGCTCGACTCAGGAGGCACATTGTAGTTAGAGCGTGCTTCT
TCTTCTCCGCACTCTTCTCGCTTCCTCCACCCCCTCCGACCACACAACACC
GCCTTTCGCTCTGTTGTGTCTGTTGTCA

APPENDIX 7

Appendix 7.1 Blast of closest species from GenBank homologue to isolated bacteria from the anaerobic FBBR inoculated with *Enterobacter cloacae* and *Citrobacter freundii*

Sample 2

Accession number	Description	Max identity	Reference
DQ816392.1	Uncultured bacterium clone aab17e04 16S ribosomal RNA gene, partial	99%	Rawls *et al.* (2006)
EF679196.1	Uncultured *Citrobacter sp*. clone ASP-42 16S ribosomal RNA gene, partial sequence	99%	Kim (2005)
AJ233408.1	*Citrobacter freundii* 16S rRNA gene (strain DSM 30039)	99%	Sproer *et al.* (1999)

Sample 3

Accession number	Description	Max identity	Reference
DQ816391.1	Uncultured bacterium clone aab17e04 16S ribosomal RNA gene, partial sequence	97%	Rawls *et al.* (2006)
EF491825.1	*Citrobacter sp. F1-1* 16S ribosomal RNA gene, partial sequence	97%	Wildschutte & Lawrence (2007)
AJ853891.1	*Citrobacter freundii* partial 16S rRNA gene, strain WAB1942	97%	Savelieva *et al.* (2004)

Sample 4

Accession number	Description	Max identity	Reference
DQ817602.1	Uncultured bacterium clone aaa64a08 16S ribosomal RNA gene, partial	93%	Rawls *et al.* (2006)
AJ853891.1	*Enterobacter ludwigii* 16S rRNA gene, type strain EN-119T	93%	Hoffmann *et al.* (2005)

Sample 5

Accession number	Description	Max identity	Reference
DQ192061.1	*Citrobacter sp*. I101-10 16S ribosomal RNA gene, partial sequence	98%	Kim (2005)
EF669481.1	*Citrobacter freundii* strain HC050630B-1 16S ribosomal RNA gene, partial sequence	98%	Fang *et al.* (2007)

Sample 6

Accession number	Description	Max identity	Reference
DQ192061.1	*Citrobacter sp.* I101-10 16S ribosomal RNA gene, partial sequence	100%	Kim (2005)
EF669481.1	*Citrobacter freundii* strain HC050630B-1 16S ribosomal RNA gene, partial sequence	99%	Fang *et al.* (2007)

Sample 7

Accession number	Description	Max identity	Reference
AJ431329.1	*Bacillus sp. ikaite c1* partial 16S rRNA gene, isolate ikaite c1	98%	Stougaard *et al.* (2002)
AF260711.1	*Bacillus sp*. S4 16S ribosomal RNA gene, partial sequence	98%	Galkin *et al.* (2000)
X68415.1	*B.globisporus* gene for 16S rRNA	98%	Ludwig *et al.* (1992)

Sample 8

Accession number	Description	Max identity	Reference
AB326543.1	Uncultured bacterium gene for 16S rRNA, partial sequence	97%	Isobe *et al.* (2007)
EF469213.1	*Pantoea sp.* KF20 16S ribosomal RNA gene, partial sequence	97%	Adesina *et al.* (2007)
AJ853891.1	*Enterobacter ludwigii* 16S rRNA gene, type strain EN-119T	97%	Hoffmann *et al.* (2005)
AM491469.1	*Enterobacter sp*. Nj-68 16S rRNA gene, strain Nj-68	97%	Gai (2007)

Appendix 7.2 Blast of closest species from GenBank homologue to isolated bacteria from the anaerobic FBBR inoculated with activated sewage sludge

Sample 2

Accession number	Description	Max identity	Reference
AY976809.1	Uncultured bacterium clone K427 16S ribosomal RNA gene, partial sequence	85%	Eckburg *et al.* (2005)
EF117251.1	Bacteroidetes symbiont of Osedax sp. 16S ribosomal RNA gene, partial sequence	83%	Goffredi *et al.* (2007)
AY298788.1	Flavobacteriaceae bacterium G812M2 16S ribosomal RNA gene, partial	83%	Abell *et al.* (2005)

Sample 3

Accession number	Description	Max identity	Reference
DQ307723.1	Bacterial diversity in intestinal tract of the fungus cultivating termite Macrotermes michaelseni Sjoestedt	100%	Mackenzie *et al.*, (2005)

Sample 4

Accession number	**Description**	**Max identity**	**Reference**
EF515599.1	Uncultured bacterium clone 30g04 16S ribosomal RNA gene, partial sequence	91%	Dryden *et al.* (2007)
AJ005635.1	Phylogeny of *Prevotella enoeca* and *Prevotella tannerae*	82%	Downes *et al.* (1998)
AF018521.1	Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes	82%	Whitford *et al.*(1997)
AF544206.1	Rumen bacterium YS1 16S ribosomal RNA gene, partial sequence	82%	Shin *et al.* (2002)