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Thermophilic anaerobic biohydrogen production from cellulosic materials

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THERMOPHILIC ANAEROBIC BIOHYDROGEN PRODUCTION FROM CELLULOSIC MATERIALS

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A research report submitted to the Faculty of Science, University of the Witwatersrand, fulfilling the requirements for the degree of Master of Science (Biotechnology)

Johannesburg 2010

DECLARATION

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



_____

(Rosario Tizzone)

22nd day of July 2010

ABSTRACT

The unabated exploitation of fossil fuel sources to provide the world with its energy demands has resulted in a dangerous increase in greenhouse gases and consequently global warming. Due to increased awareness and public concern increasing amounts of research has been devoted to identifying and developing alternative fuel sources into sustainable processes for energy production.

Hydrogen is highly attractive due to its unrivalled energy density per unit mass. This present study aimed to produce hydrogen from biological sources (biohydrogen) through the process of dark fermentation. Wheat-bran was used as the fermentable carbohydrate substrate. The bioreactor system in which the experiment was conducted selected for anaerobic, thermophilic, cellulolytic hydrogen-producing bacterial consortia derived from sewage sludge.

The results of this study showed variable rates of hydrogen production, ranging from 0.37 L/(L.h) to 0.78 L/(L.h). Various bioreactor parameters were monitored and it was found that hydrogen production was dependent on an optimal pH range between 5.0 and 6.0. The bioreactor proved difficult to maintain in terms of parameter conditions despite being an overall successful achievement of hydrogen production. The possibility of this bioprocessor being up-scaled into an industrially viable biofuel generator remains bleak as the energy input required to maintain optimal hydrogen production exceeds the chemical energy outputs of biohydrogen.

DEDICATION

Cui cerca, trova; cui sècuta, vinci.

Who seeks, finds; who perseveres, wins.

To my family,

My parents, Ursula and Angelo

My brother, Miguel

For their love and support throughout my academic career

The foundations they have set have made me into the man I am today

I will always be grateful

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3. To the love of my life, Sofi, for her unconditional commitment and support throughout my university tenure and especially during this last year of my studies.

4. Finally to the Gauteng Department of Agriculture, Conservation and Environment (GDACE) for providing the funding for my studies.

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LIST OF ABBREVIATIONS

acetyl-CoA	-	Acetyl- Co-enzyme A
AFBR	-	Anaerobic fluidised bed reactor
ATP	-	Adenosine tri-phosphate
BEAMR	-	Bioelectrochemically assisted microbial reactor
CIGSB	-	Carrier induced granular bed bioreactor
COD	-	Chemical oxygen demand
CSTR	-	Continuous stirred tank reactor
DGGE	-	Denaturing gradient gel electrophoresis
dH_2O	-	Distilled water
DNA	-	Deoxyribonucleic acid
Fd	-	Ferredoxin
Fhl	-	formate:hydrogen lyase
GC	-	Gas chromatograph
H_2ase	-	Hydrogenase
HRT	-	Hydraulic retention time
LCFA	-	long chain fatty acid
N_2ase	-	Nitrogenase
NADH	-	Reduced nicotinamide adenine dinucleotide
PCR	-	Polymerase chain reaction
Pfl	-	pyruvate**:**formate lyase
Pfor	-	pyruvate:ferredoxin oxidoreductase
PHP	-	Potassium hydrogen phthalate
PSI	-	Photosystem I
PSII	-	Photosystem II
UASB	-	Upflow anaerobic sludge bed

LIST OF UNITS AND SYMBOLS

%	-	Percentage
% v/v	-	Percentage volume per volume
°C	-	Degrees centigrade
µL	-	Micro litre
bp	-	Base pair
cm^3	-	Cubic centimetre
e^-	-	Electron
g	-	Gram
h	-	Hour
K_1 / K_2	-	Equilibrium constants
Kg	-	Kilogram
KJ	-	Kilojoule
L	-	Litre
min	-	Minute
mL	-	Millilitre
mM	-	Milli molar
mm	-	Millimetre
mol	-	Mole
mV	-	Milli volt
N	-	Nitrogen
nm	-	Nanometer
R	-	Universal gas constant
rpm	-	Revolutions per minute
s	-	Second
T	-	Temperature
V	-	Volt
ΔG°	-	Gibbs free energy
ΔH°	-	Enthalpy

1. INTRODUCTION

With fossil fuel reserves diminishing and the effects of anthropogenic climate change evident, the world is in need of radical change in order to sustain the rapidly growing human populations. The process of obtaining, processing and combusting fossil fuels has contributed significantly to global warming with CO_2 emissions accounting for 60 % of the greenhouse gases (Carere *et al*., 2008). Increasing populations means increased energy consumption, and it is estimated to increase by 54 % within the next 15 years (Carere *et al*., 2008). Thus the problem is evident considering the lack of sustainability of current energy sources and the processing thereof.

Taking a retrospective view into the history of mankind's endeavours to create fuels that drive our race, it is clear that an evolutionary trend exists towards more efficient, cleaner, sustainable energy sources. From wood to coal and petroleum, to methane and eventually towards hydrogen, there is an urgent necessity to develop alternative energy sources that can replace our dependence on fossil fuels (Das, 2009). Of the most viable alternative energy sources currently known, hydrogen is indeed the most promising, both environmentally and economically. Hydrogen possesses the highest energy content per unit weight of any known fuel source (142 KJ/g) (Das, 2009) making it extremely attractive. Furthermore, it is not a greenhouse gas and when combusted, produces water vapour which can be recycled again to produce hydrogen. It is also less volatile than natural gas making it less hazardous and thus easily transportable via conventional means (Das, 2009).

Currently, hydrogen generation is primarily fossil-fuel derived, with 30 % from oil and naphtha and 18 % from coal (Nath and Das, 2003; Suzuki, 1982). More specifically, these processes are: steam reformation and thermal cracking of natural gas; partial

oxidation of naphtha hydrocarbons; and gasification of coal (Das and Veziroglu, 2001), with high temperature steam reformation being by far the most used (90 %) (Rosen and Scott, 1998; Lodhi, 1987). Important to note however, is that these processes utilise fossil-fuel-derived energy and are highly energy intensive (Rosen and Scott, 1998), consequently they are not sustainable processes. Researchers have thus turned to favour biological hydrogen production.

1.1. Biohydrogen

Approximately 1 % of hydrogen is derived from biological sources (biohydrogen) and presents a highly attractive avenue for research with unparalleled flexibility as a sustainable energy system that is virtually inexhaustible (Greenbaum, 1990; Sasikala *et al*., 1993; Benemann, 1997; Das and Veziroglu, 2001). Biohydrogen production is catalysed by microorganisms that do not necessitate extremes of pressure or temperature (Das and Veziroglu, 2008), contrasting the fossil-fuel based processes. Moreover generating biohydrogen does not require massive power plants and can be achieved in decentralised smaller-scale systems, where suitable biomass is available and effectively eliminates transport costs and reduces unnecessary energy expense. Biohydrogen is produced via several routes: Water biophotolysis; photofermentation; dark fermentation; and a mixture of fermentative and photosynthetic bacteria functioning in a hybridised metabolic system (Das and Veziroglu, 2001).

1.1.1. Water biophotolysis

Photosynthetic algal species and cyanobacteria are capable of splitting water molecules in this process and it is essentially photosynthesis except instead of generating carbohydrates, hydrogen is produced. The photosystems that facilitate photosynthesis function serially, with hydrolysis and O_2 generation occurring at photosystem II (PSII) and CO_2 reduction at PSI. The electrons removed from water molecules this way drive

the reduction of CO_2 or the formation of H_2. Plants do not produce H_2 due to the absence of hydrogenase enzymes and are subsequently only capable of the reduction of CO_2 (Ramachandran and Menon, 1998). Microalgal species (eukaryotic green algae and prokaryotic cyanobacteria) however do possess hydrogenases and as such can produce hydrogen in particular circumstances (Benemann, 1997).

Since the first successful experiment showing microalgal hydrogen photo-evolution in 1992 by Gaffron and Rubin, there have been several research groups that have scrutinised the mechanistic behind this system. It is known that during biophotolysis, electrons travel from water through the photosystems to hydrogenase mediated by an electron carrier called ferredoxin (Fd) and that when there is a presence of O_2, hydrogen production is greatly reduced (Benemann *et al*., 1973). It is now known that various green algae species produce basal amounts of hydrogen in the dark, under anaerobic conditions and upon light exposure (maintaining anaerobic conditions), hydrogen evolution is greatly increased, although once normal photosynthesis is re-established (O_2 production and CO_2 fixation), hydrogen evolution halts (Das and Veziroglu, 2001).

Cyanobacteria and blue-green algae are nitrogen-fixing bacteria capable of biophotolysis using hydrogenase and another hydrogen-evolving enzyme called nitrogenase (Smith *et al*., 1992). Figure 1a (Hallenbeck and Ghosh, 2009) illustrates a summary of biophotolysis with the hydrogen-catalysing enzymes, hydrogenase and or nitrogenase depending on the organism involved. A major drawback to this system as a potential biohydrogen-generator is the O_2-sensitivity of hydrogenase and nitrogenase which are irreversibly inactivated when O_2 evolution is active (Lambert and Smith, 1980; Sarker *et al*., 1992).

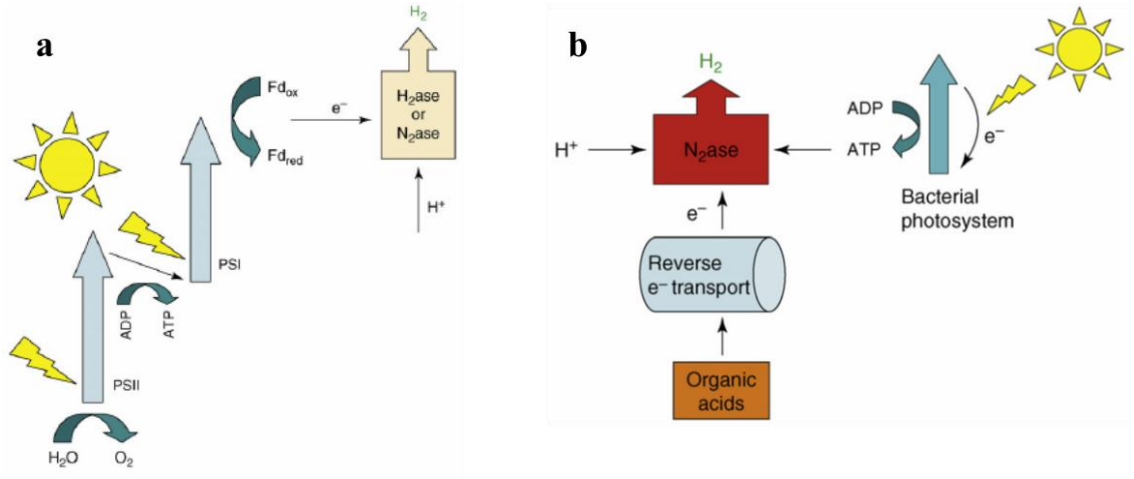


Figure 1.1. (a) Biophotolysis occurring in a photosynthetic system, depicted are photosystems I and II (PSI and PSII), the electron carrier, ferredoxin (Fd) and the hydrogen-evolving enzymes, hydrogenase and nitrogenase (H_2ase and N_2ase) (b) The photofermentation process whereby organic acids act as electron donors, which follows reverse electron transport and together with ATP generated by light energy, nitrogenase reduces the proton to hydrogen (Hallenbeck and Ghosh, 2009).

1.1.2. Photofermentation

The photo-decomposition of organic compounds by phototrophic bacteria has shown great potential as a biohydrogen production system (Kim *et al.*,1981; Vincezini *et al.*, 1982; Miyake and Kawamura, 1987; Fascetti *et al.*, 1998). The purple non-sulphur bacteria are photofermenters and produce hydrogen by absorbing light and fermenting reduced compounds such as organic acids (Bolton, 1996; Fedorov *et al.*, 1998). These anaerobic photoheterotrophic bacteria do not possess PSII and as a result do not produce O_2, thus there is no inhibition in hydrogen production. Furthermore, they are able to utilise a variety of organic and inorganic substrates as electron donors (as opposed to water, by photoautotrophs), and a number of studies corroborate this in different experimental environments, including batch processes (Zurrer and Bachofen, 1979) continuous cultures (Fascetti and Todini, 1995) as well as immobilised whole cell systems with various solid support matrices (Francou and Vignais, 1984; Vincenzini *et al.*, 1986; Fedorov *et al.*, 1998).

The photofermentative process essentially involves solar energy being captured and utilised to produce ATP and release electrons via reverse electron flow, which reduces

ferredoxin, and together with ATP, drive hydrogen evolution via proton reduction carried out by nitrogenase. Figure 1b (Hallenbeck and Ghosh, 2009) illustrates this process. Photofermentation bears numerous advantages over biophotolysis, the most significant include, high theoretical conversion yields; absence of O_2-evolving capabilities therefore no hydrogenase or nitrogenase deactivation; and the ability to utilise organic wastes as substrates (Das and Veziroglu, 2008).

1.1.3. Dark fermentation

This report is based on experiments exploiting dark fermentation as a process to produce biohydrogen and as such, the principles regarding this metabolism need to be explained in greater detail than the aforementioned methods. Bacteria growing anoxically and heterotrophically are able to oxidise various organic substrates providing the necessary energy to promote metabolic growth (Das and Veziroglu, 2008). Contrasting aerobic metabolisms, wherein hydrogen is oxidised, and the derived electrons drive energy production, with excess electrons being accepted by external electron acceptors; in anoxic conditions, excess electrons are disposed of through proton reduction to form molecular hydrogen (Levin *et al*., 2004; Das and Veziroglu, 2008; Hallenbeck, 2009).

When considering the organic substrates that necessitate hydrogen production from dark fermentation, one is essentially limited to carbohydrate sugars, as opposed to proteins or lipids (Das and Veziroglu, 2008). This is due to the specific fermentation reactions occurring in lipids and proteins that do not produce hydrogen. In the case of proteins, hydrolysed amino acids ferment in pairs, with one being the electron acceptor for the oxidation of the second, also known as Stickland reactions (Gottschalk, 1986). With lipids, the hydrolysis thereof produces glycerol and long chain fatty acids (LCFAs), and while LCFAs can be degraded to produce hydrogen and acetate, this

process naturally only occurs by syntrophic bacteria at very low hydrogen partial pressures (McInerney *et al*., 2007). Thus dark fermentation is only amenable for hydrogen production when fermenting carbohydrate-rich materials, since the formation of pyruvate from glycolysis serves as the critical intermediate for the various pathways leading to hydrogen generation (Hallenbeck, 2005).

There are two kinds of enzymes that enable the degradation of pyruvate to subsequent intermediates for hydrogen production: pyruvate:formate lyase (Pfl) and pyruvate:ferredoxin oxidoreductase (Pfor). The former primarily involved in enteric, mixed-acid fermentation and the latter predominant in clostridial-type fermentations (Hallenbeck and Ghosh, 2009). Looking specifically at mixed-acid fermentation by enteric-type bacteria (facultatively anaerobic) schematically shown in figure 1.1 (Hallenbeck and Ghosh, 2009), pyruvate is cleaved, forming acetyl-CoA and formate. The subsequent hydrogen production derived from formate does not occur unless the formate:hydrogen lyase pathway is induced as a response to a drop in environment pH, which will then alleviate acidification by converting the formic acid to hydrogen (Hallenbeck, 2009). As the diagram in figure 3 depicts, lactate can also be produced through conversion of pyruvate and this can also reduce acidic conditions due to the production of only one acidic molecule (acetic acid) instead of the potential two acids (acetic and formic) (Hallenbeck, 2005). This reaction is unfavourable for hydrogen production as reductants are diverted away from that hydrogen-generating pathway. Considering formate, a maximum of two molecules thereof can be produced per glucose molecule and as such, a predicted maximum of two molecules of hydrogen can be generated per glucose.

The reaction occurring in Clostridia, which are obligately anaerobic bacteria, pyruvate is converted to acetyl-CoA and CO_2 resulting in the reduced ferredoxin that transfers its

electrons to a hydrogenase, consequently generating hydrogen. This process indicates two moles of hydrogen produced per mole of glucose metabolised. However, unlike enteric bacteria, Clostridia are also capable of coupling NADH oxidation with proton reduction to form hydrogen (Hallenbeck, 2005). Although this only takes place in low partial pressures of hydrogen and as yet, the precise details of the path that is followed have yet to be delineated. Thus four moles of hydrogen can be produced per mole of glucose if acetate is the final organic acid product and this is shown by the chemical formula:

$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$

This is the maximum amount of hydrogen that can be generated by dark fermentation, of course this is only theoretical and in practice, there are numerous factors influencing hydrogen production (Levin *et al*., 2004). The diagram shown in figure 1.2 (Ren *et al*., 2006) gives a much clearer understanding of the dark fermentation pathways that can be followed. Pyruvate is the central molecule from which all the other organic acid pathways evolve. Lactic acid can be produced directly from pyruvate, which drives electron flow away from hydrogen generation, hence for biohydrogen production, it is avoided. Contrasting this, and as explained above, acetic acid generation, is the ideal pathway from which ferredoxin can drive hydrogen evolution. If any other organic acid is produced as the final product, hydrogen ions are consumed in the process, thus lowering overall hydrogen yield. Therefore in bioprocesses exploiting dark fermentation, considerable measures are taken to ensure fermentation is directed towards more volatile fatty acid production (like acetic acid). Practically this is very difficult to achieve, although higher hydrogen yields are associated with acetate and butyrate fermentation products, while if propionate, ethanol or lactic acid are the end products, hydrogen yields are much lower.

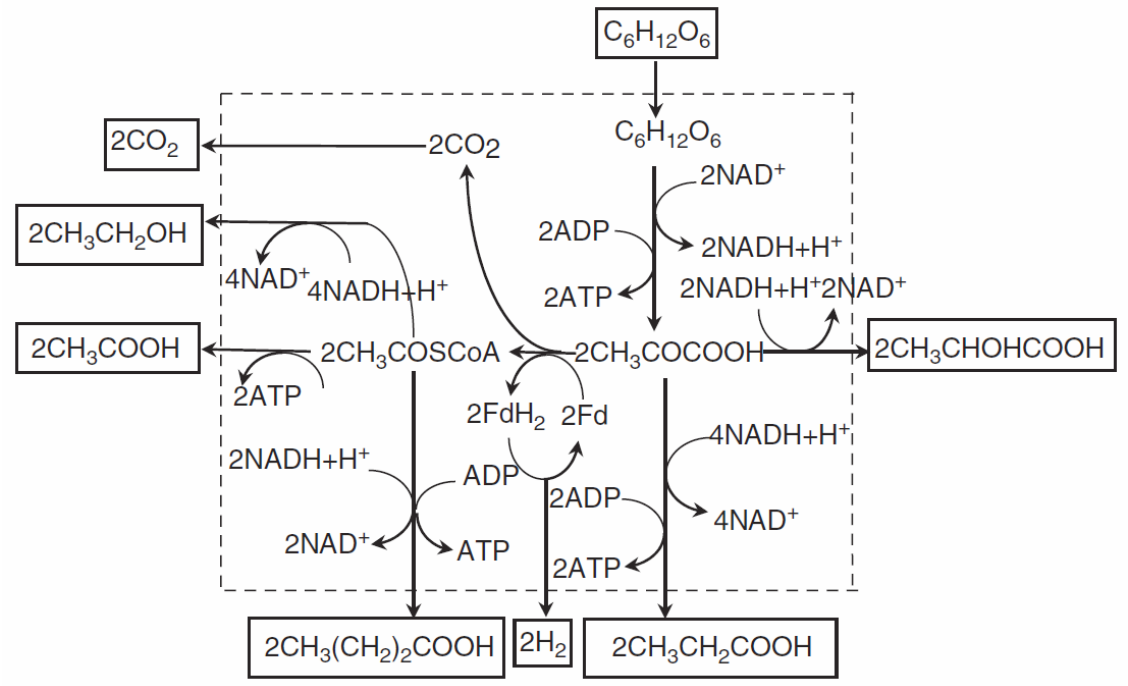


Figure 1.2. Summary of glucose fermentation pathways that can be engaged with hydrogen production optimised when the system is driven towards acetic acid generation (Ren *et al*., 2006).

Dark fermentation does however yield relatively low amounts of hydrogen with respect to the costs of the carbohydrate substrates (glucose and sucrose). It is known that the productivity and yield of hydrogen is fundamentally affected by the process conditions in which the bacteria are exposed.

Aside from the extrinsic factors that influence biohydrogen production, the types of cultures that can be used for fermentation have also varied. There is an abundance of literature showing successful experiments using pure bacterial cultures to produce hydrogen, primarily with *Clostridium* and *Enterobacter* species (Li and Fang, 2007). The review by Wang and Wan (2009) lists a comprehensive collection of pure cultures that have been researched and the level of hydrogen production associated therewith.

Turning to mixed bacterial cultures, the bacteria capable of dark fermentation are essentially ubiquitous and hydrogen-producing mixed cultures have been obtained from varied environments including soil, wastewater sludge and manure (Cheong and Hansen, 2006; Zhu and Beland, 2006; Hu and Chen, 2007; Wang and Wan, 2008). In

using mixed cultures over pure cultures, to produce hydrogen, various practical issues are addressed and this makes the former more attractive. These include easier operation and control as well as possessing a dynamic range of feedstock options (Li and Fang, 2007). Additionally mixed cultures can be compelled into aggregations of macroscopic flocs or granules which, by contrast with pure culture, are relatively easier to achieve. The phenomenon of granulation is fundamentally important factor in anaerobic fermentations and will be discussed in greater detail in the bioprocess parameters section.

Another advantage with using mixed consortia is the fact that at industrial scale, fermentations will be carried out in non-sterile conditions, which may prove inhibitory to pure cultures and this is precisely what microbial consortia have been selected for in their respective non-sterile environments (Hallenbeck and Ghosh, 2009). Furthermore the nature of microbial consortia to function synergistically facilitates their elevated resilience, compared to pure cultures, towards altered environmental conditions (Kleerebezem and Loosdrecht, 2007). Moreover, a diverse microbial community possesses a much wider metabolic range than a pure culture would and as such will be capable of degrading an increased variety of suitable substrates. This situation can be problematic though, considering that while certain bacteria may be producing hydrogen, others may be consuming it. For this reason, it is imperative that more research goes into understanding the interactions that occur within microbial populations, and from that knowledge construct tailored consortia optimised metabolically for the synergistic production of hydrogen.

It has been found that hydrogen-producing bacteria may possess certain traits that allow them to remain viable in harsher conditions and this has been exploited in bioprocessing in the form of various pre-treatment methods (Reviewed in Wang and

Wan, 2008). The predominating enrichment methods are heat-shock and acid treatments although the method chosen largely depends on the type of mixed culture and the choice of fermentative bioprocess to follow (Li and Fang, 2007). There are naturally some discrepancies amongst researchers as to which pre-treatments are the most effective at enriching for hydrogen-producers and it ultimately boils down to the highest level of hydrogen production for a given fermentative process (Cheong and Hansen, 2006; Zhu and Beland, 2006; Hu and Chen, 2007; Mohan *et al*., 2008; Wang and Wan, 2008). Bearing in mind that most of the comparisons of pre-treatments have been done on bioprocesses running in batch and using sucrose or glucose feedstock; therefore extra consideration must be given towards continuous bioprocesses that utilise organic wastes as feed substrates, since it is these experiments that will essentially pioneer fermentative-biohydrogen technology into industrialisation.

1.1.4. Hybridised systems

In the hope to improve hydrogen yields efficiently and economically, hybrid systems have been developed which attempt to enhance overall hydrogen production by combining the metabolism of non-photosynthetic and photosynthetic bacteria (Das and Veziroglu, 2001). Thus bacteria capable of dark fermentation will utilise the carbohydrate sources for the production of organic acids. These can then be completely degraded by photofermentative bacteria to hydrogen and carbon dioxide, contributing to a total net increase of hydrogen. Represented below are the chemical formulae delineating the process:

Stage I: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$

Stage II: $2CH_3COOH + 4H_2O \rightarrow 8H_2 + 4CO_2$

In this case, a theoretical total of 12 moles of hydrogen can be obtained from one mole of glucose, provided acetic acid is the predominating metabolite from dark fermentation. There have been a number of studies looking at hybridised systems including Lee *et al*. (2002) who exploited effluent wastewater for hydrogen production using purple non-sulphur bacteria along with anaerobic bacteria. Another study by Kim and colleagues (2001) employed the same metabolic combination to optimise hydrogen productivity from sewage sludge and food processing wastewater; while Nath and Das (2005) used glucose as a dark fermentation substrate and the derived effluent as a substrate for photofermentation.

Using bioreactor microbial fuel cells as a hybrid system to generate hydrogen from organic matter oxidation has also been looked at (Schotz and Schroder, 2003; Ishikawa *et al*., 2006). Protons and electrons generated by active bacterial metabolism create an electrical current between the anode and cathode. Using a bioelectrochemically assisted microbial reactor (BEAMR), hydrogen can be generated at the cathode by eliminating oxygen and applying a voltage (Liu *et al*., 2005), depicted by the following formulae:

$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$

Anode: $CH_3COOH + 2H_2O \rightarrow 2CO_2 + 8e^- + 8H^+$

Cathode: $8H^+ + 8e^- \rightarrow 4H_2$

In this case 8 moles of hydrogen can be generated per mole of glucose consumed following acetate production. This process is considerably more energy efficient as compared to methods of water electrolysis, which require around 1.8 – 2.0 V to catalyse hydrogen production, whilst in the BEAMR, bioelectrolysis is achieved at approximately 0.25 V (Liu *et al*., 2005). For further reading on microbial fuel cells refer to Schotz and Schroder (2003) as well as Liu *et al*. (2005).

1.2. Bioreactors

Batch reactors demonstrate the principles of fermentative hydrogen production easily due to their relatively simple operation and control. However to appreciably realise the industrial potential of biohydrogen production, continuous reactor systems need to be used. There is a literature deluge with the number of studies conducted using continuous bioreactor systems in various configurations, with continuous stirred tank reactors (CSTRs) predominating (Chen and Lin, 2003; Ting *et al*., 2004; Gavala *et al*., 2006; Zhang *et al*., 2006; Lee *et al*., 2007; Wu *et al*., 2008). Bacterial biomass contained within CSTRs is well suspended in the solution and thus possess the same retention time as the hydraulic retention time (HRT). This means that biomass washout will occur at lower HRTs and moreover there is a limited concentration that biomass can achieve in suspension and by extension limited hydrogen production (Wang and Wan, 2008). By creating an immobilisation matrix to which biomass can attach, effectively prevents washout at low HRTs while also increasing effective biomass concentration as well as hydrogen production (Li and Fang, 2007).

Various methods have been employed in immobilising biomass in the forms of biofilms and granules. Zhang *et al*. (2008) compare biohydrogen production within anaerobic fluidised bed reactors using either biofilms or granules and their data show that granule-based bioreactors are more efficient for continuous fermentative hydrogen production due to enhanced biomass retention. The maintenance of these high volumetric biomass densities under high dilution rates without significant biomass washout is a highly advantageous trait that fluidised granular bed bioreactors have over their predecessors. This shows a gradual augmentation of the bioreactor technology, since all fluidised granular bed bioreactors are essentially just modified versions of the traditional upflow anaerobic sludge bed (UASB) bioreactors (Stronach *et al*., 1986).

A modified anaerobic fluidised bed bioreactor was constructed for use in the experiments of this report primarily for its enhanced granule induction and growth protocols (Lee *et al*., 2006; Zhang *et al*., 2007, 2008). In spite of the superiority of granule-based bioreactor systems, the assimilation of this biohydrogen biotechnology has been sluggish. Publication of the dramatic increases in hydrogen productivities (7.3 – 9.3 L H_2/(L.h), Lee *et al*., 2006) that have been achieved with this type of reactor system have had minimum impact on the broader biohydrogen bioreactor development research agenda. The apparent reluctance to accept granule-based bioreactor systems notwithstanding, the Bioprocess Research Lab at the University of the Witwatersrand has further developed on this technology and successfully reproduced the work of Lee *et al*, (2006) and Zhang *et al*, (2008) for thermophilic bacterial consortia. There are numerous parameters that are considered for the optimisation of biohydrogen production in an anaerobic fluidised bed bioreactor and these are described briefly in the following section.

1.2.1. Bioprocess parameters

Nitrogen and phosphate

Of the numerous factors influencing optimal biohydrogen production in a bioreactor, nutrition is the most fundamental, and the provision of ammonia nitrogen and phosphate in the nutrient media for bacterial growth. Nitrogen is essential for protein and nucleic acid synthesis, therefore is indispensible for bacterial metabolism and subsequent hydrogen production (Bisaillon *et al*., 2006). The precise amount of ammonia nitrogen required for optimal growth remains a question of much disparity with numerous studies showing diverse results; Bisaillon and colleagues (2006) reporting 0.01 g N/L ammonia nitrogen while Salerno *et al*. (2006) reported 7.0 g N/L. Of course this disagreement can be attributed to the different inocula used and the

ammonia concentration ranges studied. A number of studies that have researched the effect of ammonia nitrogen concentration on fermentative hydrogen production have used glucose as a carbon source and were run in batch mode (Lin and Lay, 2004; Argun *et al*., 2008; O-Thong *et al*., 2008). While this is useful, further investigation using organic wastes as substrates and running continuously, will give a more accurate representation of the metabolic requirements of ammonia nitrogen in hydrogen-producing bacterial cultures in systems amenable for up-scaling.

Phosphates also possess high nutritive value for bacterial growth as well as a capacity for solution buffering (Wang and Wan, 2008). Varying the concentration of phosphates influences bacterial growth and consequently hydrogen production (Bisaillon *et al*., 2006). Investigations into the optimal ratios for nitrogen and phosphate to carbon source have shown that there are indeed critical proportions for each, however these vary greatly among the respective studies (Lin and Lay, 2004; Argun *et al*., 2008; O-Thong *et al*., 2008). This again illustrates the necessary requirement for comprehensive studies with consistent bioreactor conditions that can give a better, accurate depiction of the optimum metabolic requisites for hydrogen production.

Additional mineral salts

Hydrogen production is also influenced by the mineral salt composition of the nutrient media and Lin and Lay (2005) reported an optimised mineral salt composition comprising various proportions of $CaCl_2 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot 7H_2O$, KI, $KMnSO_4 \cdot 4H_2O$, $MgCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 6H_2O$, NaCl, $Na_2MoO4 \cdot 2H_2O$, NH_4Cl, $NiCl_2 \cdot 6H_2O$ and $ZnCl_2$; that resulted in 66 % greater hydrogen production rates than conventional acidogenic nutrient solution (NH_4HCO_3, K_2HPO_4, $MgCl_2 \cdot 6H_2O$, $MnSO_4 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $CoCl_2 \cdot 5H_2O$, $NaHCO_3$). Additionally, the trace metals, iron, magnesium, sodium and zinc were

found to be essential factors affecting hydrogen productivities due to their integral roles in enzyme co-factors, hydrogenase enzymes and various transport processes (Davila-Vazquez *et al*., 2007).

pH

Depending on the pH of the bioreactor, hydrogenase activity can be adversely affected and more importantly, the entire metabolism can shift from acidogenic, to methanogenic activity (Wang and Wan, 2008). Optimum pH levels have been found to differ depending on the particular process conditions, however several groups have shown maximum hydrogen production rates between pH 5.0 and 6.0 (Reviewed in Davila-Vazquez *et al*., 2007). Methanogenic activity is effectively inhibited at moderately acidic pH coupled with high temperatures (Oh *et al*., 2004; Kotsopoulos *et al*., 2006). In batch systems, it was found that organic acid production lowered overall medium pH due to reduced buffering capacity (Davila-Vazquez *et al*., 2007) and that volatile fatty acid (VFA) as well as ethanol production were pH dependent (Mu *et al*., 2006). Moreover, enhanced hydrogen production rates were shown to be associated with acetate and butyrate production, while, when associated with propionate formation, overall hydrogen productivities decreased (Oh *et al*., 2004; Wang *et al*., 2006).

Temperature

Both the metabolic activity and the growth rate of microorganisms are affected by temperature. Fermentative hydrogen production has been shown predominantly at mesophilic (25 – 40 °C) to thermophilic (40 – 65 °C) temperature ranges (Reviewed in Davila-Vazquez *et al*., 2007). By examining Gibbs free energy and standard enthalpy of glucose to acetate conversion, the effect of temperature on hydrogen productivity can be shown:

$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$

$\Delta G° = -176.1$ KJ/mol

$\Delta H° = +90.69$ KJ/mol

Both the Gibbs free energy and enthalpy changes show the reaction is endothermic and can occur spontaneously. Using the Van't Hoff equation, the temperature effect on the equilibrium constant can be explained (Smith *et al*., 2000):

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

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

Following equation 1, increasing the temperature results in an increased equilibrium kinetic constant due to the reaction being endothermic (positive $\Delta H°$), thus an increased temperature of glucose fermentation while maintaining constant reactant concentrations (equation 2) would result in greater hydrogen production. A study by Valdez-Vazquez *et al*. (2005) corroborates this with their results showing a 60 % greater hydrogen production rate at thermophilic versus mesophilic conditions. This could be attributed to the fact that the hydrogenase enzyme present in the thermophilic Clostridia has an optimal temperature range of between 50 and 70 °C.

By maintaining thermophilic temperatures, lactate-producing bacteria are effectively inhibited (Oh *et al*., 2004), and potential pathogenic bacterial strains are also removed (de Mes *et al*., 2003). Thermal denaturation of proteins vital to metabolic activity is an important consideration to make when working with thermophilic temperatures and higher. The study by Lee *et al*. (2006) details the effects of temperature on hydrogen

production within a carrier induced granular bed bioreactor (CIGSB) and their results showed an inhibition of granular sludge formation due to enzyme denaturation.

1.3. Project aims and objectives

The principal aim of this project was to generate high biohydrogen production rates by exploiting the dark fermentation of cellulosic materials in an anaerobic fluidised bed bioreactor system using thermophilic, cellulolytic bacterial consortia derived from sewage sludge. Several objectives were created as a plan of action to put the project into perspective. These were:

- To select and enrich anaerobic, thermophilic, cellulolytic, mixed bacterial consortia from sewage sludge.
- To establish a bioreactor that is fed-batch with regard to the cellulosic substrate (wheat bran) and continuous with regard to inorganic nutrients (mineral salts medium containing ammonia nitrogen and orthophosphate)

To establish a fluidised heterogeneous bed comprising cellulosic particles; bacteria attached to thereto; planktonic bacteria; bacterial flocs; and bacterial granules.

2. EXPERIMENTAL METHODOLOGY

2.1. Sewage inoculum pre-treatment

Sewage sludge was obtained from Olifantsvlei Municipal Sewage Treatment Plant, Johannesburg. The sludge was brought to a pH of 2 by acid treatment with 1N HCl and incubated for 24 h at room temperature. A heat shock-treatment was subsequently performed by heating the sewage sludge to 90 °C for 60 min. The sludge was pH adjusted to 7.0 with addition of modified Endo-medium. Wheat bran was added (50 g/L) to the mixture and incubated at 65 °C to enrich for anaerobic thermophilic, cellulolytic bacteria. The bacterial culture was maintained and sub-cultured in Schott bottles at 65° C until inoculated into the AFBR.

2.1.1. Nutrition

The abovementioned modified Endo-medium is a mineral salts nutrient medium, based originally on the Endo-formulation (Endo *et al*., 1982) and adapted from Lee *et al*. (2003). However, no sucrose or sodium bicarbonate ($NaHCO_3$) were added. The medium comprised (g/L of distilled water): NH_4HCO_3 5.24; K_2HPO_4 0.125; $MgCl_2 \cdot 6H_2O$ 0.1; $MnSO_4 \cdot 6H_2O$ 0.015; $FeSO_4 \cdot 7H_2O$ 0.025; $CuSO_4 \cdot 5H_2O$ 0.005; and $CoCl_2 \cdot 5H_2O$ 1.25×10^{-4}. This modified nutrient Endo-medium was used to maintain sewage cultures prior to AFBR inoculation and thereafter, pumped continuously into the bioreactor using a Watson-Marlow 505Du peristaltic pump.

Wheat bran mixed with water and pre-treated by steam explosion in an autoclave (Sturdy SA 300-VF) was used as the cellulosic substrate both during enrichment of the sewage culture and in the AFBR. For the bioreactor, an initial amount of 500 g (dry mass) wheat bran was added and subsequently, 250 g (dry mass) was added at 48 hourly intervals during the bioreactor operational period.

2.2. Bioreactor operation

A pre-pilot-scale anaerobic fluidised bed bioreactor (AFBR) was constructed from plexiglass. The volume of the AFBR totalled 41 064 cm^3 (41 L) by summation of the bioreactor column (30.97 L), gas disengager and fluid filter (9.3 L) and the pipes and connections of the system (0.77 L). Figure 2.1 illustrates the bioreactor design schematically.

At start-up, 35 L of modified Endo-medium was poured into the AFBR along with 500 g steam-exploded wheat bran slurry. Following this, the pre-treated sewage was inoculated with simultaneous addition of 200 g sucrose to enhance biomass formation and lower initial pH in the system by enhancing volatile fatty acid production. The AFBR was operated under fed-batch conditions with respect to wheat bran application and under continuous conditions with respect to supply of the modified Endo-medium. For the duration of its operation, the AFBR was run at thermophilic temperature (65 °C) by means of heated water circulating through the AFBR water jacket with temperature being maintained by a heated water bath.

Regarding the bioreactor fluid recycling, the balance of effluent from the gas-disengager was recycled back into the bioreactor via a fluid filter container. Effluent recycling through the bioreactor bed facilitated mixing and fluidisation and additionally aided in optimising gas stripping from the fluidised bed. The effluent recycle rate range with respect to the rpm of the Boyser pump was 12.5 rpm (71.43 L/h), 30 rpm (171.42 L/h), 35 rpm (199.99 L/h) and 40 rpm (228.56 L/h). Recycle rates were adjusted to those that did not result in washout of the bran bed. The HRT range applied for selection was determined from the volumetric feeding rate of the 505Du Watson-Marlow pump: 9 mL/min (80 h); 10 mL/min (70 h); 14 mL/min (50 h); 21 mL/min (32 h).

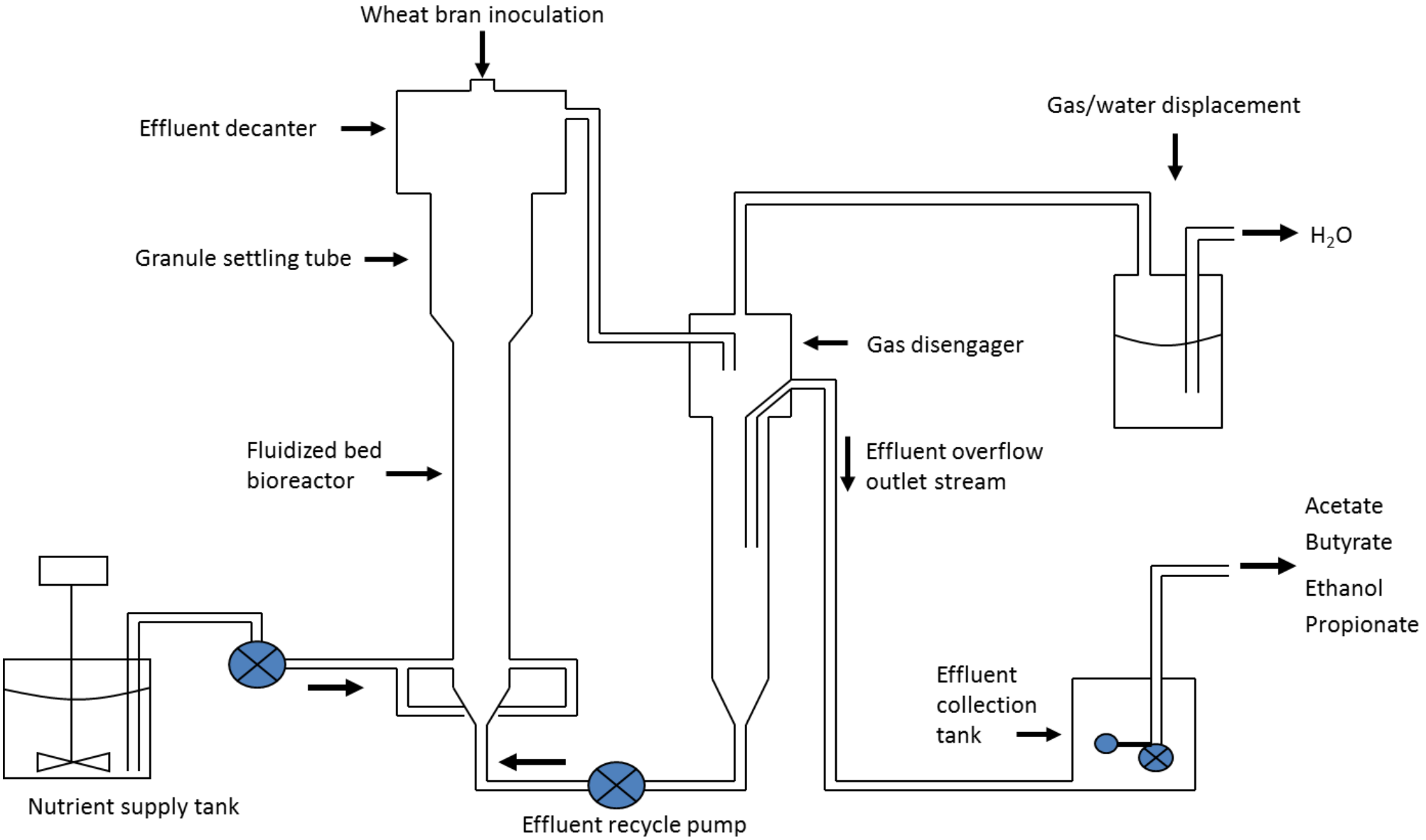


Figure 2.1. Diagrammatic representation of the bioreactor system showing inorganic nutrient supply, the primary bioreactor column, the gas/liquid disengager column, recycle pump and an effluent collection tank. Total gas production was measured via the gas/water displacement bottle. The principal metabolic by-products of the fermentation process are also shown.

2.3. Parameter monitoring and biochemical assays

2.3.1. Effluent ammonia concentration

Effluent ammonia concentrations were determined using the phenate method, based on that by Solorzano (1969) with modifications. Ten grams of phenol was dissolved in a 100 mL solution (95% ethanol and 5% propanol) to make a 10 % phenol solution. A solution of sodium nitroprusside ($Na_2Fe(CN)5NO{\cdot}2H_2O$) was made by dissolving 1 g in 200 mL distilled water (dH_2O) and stored in an aluminium-covered bottle. An alkaline solution was made by dissolving 100 g trisodium citrate dihydrate ($C_6H_5Na_3O_7{\cdot}2H_2O$) and 5 g sodium hydroxide (NaOH) in 500 mL dH_2O. A sodium hypochlorite solution (5.0 % v/v) was also made. The oxidising solution was made by mixing 100 mL of the trisodium citrate solution and 25 mL sodium hypochlorite solution together.

A standard curve was constructed using ammonium chloride (NH_4Cl) as the standard. A stock solution (10mM NH_4Cl) was made up and diluted serially into the following concentrations (mM): 0.5; 0.35; 0.25; 0.05; 0.03; 0.015; 0. Five millilitres of each solution was then dispensed into 10 mL test tubes. To this was added 0.2 mL of 10 % phenol solution, 0.2 mL sodium nitroprusside solution and 0.5 mL oxidising solution. The reaction proceeded for 60 min, after which absorbance measurements were taken at 540 nm using a Boeco S-20 Spectrophotometer (Boeco, Germany). The bioreactor samples were diluted 100 times and the procedure was repeated, replacing the diluted stock (NH_4Cl) solutions with the effluent samples.

2.3.2. Effluent hexose concentration

A modified version of the original anthrone procedure (Morris, 1948) was followed for the determination of effluent hexose concentrations. Two grams of anthrone was added

to 1 L 95 % H_2SO_4 to produce the anthrone reagent. Aliquots of 2.5 mL of each effluent sample were dispensed into 15 mL test tubes and 5 mL anthrone reagent was added thereto. The solution was mixed and allowed to stand for 10 min, after which optical densities were measured at 620 nm. A standard curve was constructed using serial dilutions of anhydrous glucose (mg/L): 10; 20; 30; 40; 50; 60 and the sample concentrations were subsequently derived.

2.3.3. Chemical oxygen demand

Based on the protocol by LaPara *et al*. (2000) a colorimetric assay was performed to determine chemical oxygen demand of effluent samples. A digestion solution and a catalyst solution were prepared. The former comprising 2.6 g potassium dichromate ($K_2Cr_2O_7$) and 8.33 g mercuric sulphate ($HgSO_4$) dissolved in 42 mL 95-99 % H_2SO_4 , to which 208 mL dH_2O was carefully added to complete the solution. The catalyst solution consisted of 5.06 g silver sulphate (Ag_2SO_4) added to 500 mL 95-99 % H_2SO_4.

The standard used for this assay was potassium hydrogen phthalate (PHP), initially prepared by dissolving 765 mg PHP into 1 L dH_2O which is equivalent to 900 mg COD/L. This was then serially diluted to the following concentrations (mg COD/L): 50; 100; 250; 500; 750. Two millilitres of each standard sample was dispensed into test tubes containing 1.5 mL digestion solution and 3.5 mL catalyst solution. These were mixed and placed onto a heating block (HI839800 COD Reactor, Hanna Instruments) for two hours at 150 °C. After cooling, absorbance was measured at 600 nm. A similar procedure was followed for the bioreactor samples, however, soluble COD and insoluble COD needed to be determined, thus for each sample, a centrifuged supernatant and an uncentrifuged solution were assayed. Ammonia and hexose concentrations were converted to COD concentrations using conversion values obtained from Anderson and Ingram (2003), a representative concentration of the overall

biochemical content could be calculated. The biomass COD was approximated from the difference between the insoluble and soluble COD concentrations. By subtracting ammonia and hexose CODs from the soluble COD, an estimated concentration of volatile fatty acids (VFAs) was attained.

2.3.4. Biogas production

Liberated biogas from the AFBR (gas-disengager) was measured by attaching a gas/water displacement bottle (Duran-Schott, Germany) to the system, whereby gas inflow displaced the liquid volume in the bottle into a 1 L measuring cylinder.

2.3.5. Hydrogen composition

The percentage hydrogen content of the total biogas produced by the AFBR was measured by gas chromatograph (Clarus 500 GC, Perkin Elmer Inc.). Hydrogen analysis was conducted using a column of length 30 m, inner diameter of 0.32 mm and maintained at a temperature of 45 °C. A 40 µL gas volume was injected from the bioreactor sample into the GC injection cap that was set to 250 °C. Gas was detected by the equipped thermal conductivity detector at a temperature of 200 °C with argon used as the carrier gas.

2.4. Molecular analyses

2.4.1. 16S rDNA amplification

AFBR samples were taken at periodic intervals and bacterial DNA was extracted using the Fungal/Bacterial DNA Kit (Zymo Research, USA). The extracted DNA was subjected to PCR using primers specific for the 16S rDNA region of the bacterial chromosome. The primers (Fermentas Life Sciences, Canada) used for this PCR were: forward - EUB968F (5´ - CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG

GCA CGG GGG GAA CGC GAA GAA CCT TAC - 3´) and reverse – Univ1392R (5´ - ACG GGC GGT GTG TRC - 3´).

The GeneAmp® PCR System 2700 (Applied Biosystems) thermal cycler was utilised for the amplification, following a 35 cycle programme comprising: 95 °C (3 min); 94 °C (30 s); 60 °C (45 s); 72 °C (90 s) and 72 °C (7 min). The PCR products were visualised on a 1 % agarose gel electrophoresed for 35 min at 90 mV. The O'Gene Ruler™ 100 bp DNA Ladder Plus (Fermentas Life Sciences, Canada) was used as the DNA size reference.

2.4.2. Denaturing gradient gel electrophoresis

DGGE was performed to establish a microbial profile of the PCR products using the Bio-Rad Universal Mutation Detection System Dcode™, following the protocol associated therewith. A 6 % polyacrylamide gel was prepared with a denaturing gradient made from 60 % and 20 % denaturing solution. The gel was electrophoresed at 120 V at 65 °C for 3 hours after which it was removed and immersed in 250 mL of TAE buffer containing 3 µL ethidium bromide for 20 min. The gel was visualised using the Bio-Rad Gel-doc XR Imager and the associated Quantity One software programme.

The DNA bands were spliced out from the gel on a UV trans-illuminator (Fotodyne Inc.) and placed into individual 1.5 mL Eppendorf tubes. A 100 µL aliquot of TE buffer was added thereto, facilitating DNA migration from the gel fragment into the buffer solution. This was incubated overnight at 4 °C and the DNA was electrophoresed to confirm fragment fidelity and concentration. The purified fragments were sent for sequencing analysis at the University of Stellenbosch and we are awaiting the results thereof.

3. RESULTS

The AFBR was in operation for 33 days before it was shut down. Data was obtained by analysing effluent samples over 1 – 2 day intervals over a total period of 31 days. The final results of these analyses are panel-graphed in figure 3.1.

3.1. HRT selection

The initial feeding rate of influent rate of the modified Endo-medium was approximately 21 mL/min and this was found to be too high when the effluent recycle rate was 25 rpm, the combined contributory effects of the influent and effluent recycle rates on the volume fluxes through the bioreactor resulted in an overflow of wheat bran and bacterial biomass out of the main bioreactor, consequently causing a blockage of the recycle fluid filter container. Subsequent feeding rates were lowered to prevent excess clogging and allow for biomass establishment within the reactor bed. This lowered feeding rate corresponds to the increased HRT (32 h) shown in figure 3.1 (k). The HRT was adjusted to optimise for hydrogen production and through the time course it was found that the average hydrogen productivities were greatest at HRTs of 70 – 80 h. Therefore the AFBR was maintained with an HRT of 70 h for a large proportion of the operational period and once hydrogen production levels began decreasing, the HRT was increased to 80 h with the recycle rate at 170 L/h. Operations were terminated shortly thereafter, although hydrogen productivities were shown to be on a gradual increase during that time.

3.2. Biohydrogen production

Hydrogen productivities were found to be at their highest approximately 2-5 hours post-wheat bran addition. With reference to figure 3.1 (a-d), the results showed gas production increasing steadily during the first week and peaked at slightly over 30 L/h

(Figure 3.1 c); with a concomitant increase in hydrogen production, reaching approximately 70 % at its zenith on day 6 (Figure 3.1 b). Thereafter, hydrogen productivities and total gas production varied with erratic peaks and troughs although these did correlate somewhat to pH level (Figure 3.1 a) with reduced hydrogen production at a higher pH, and enhanced when pH was within an optimal range of 5.0 – 6.0. The highest hydrogen percentage was obtained on day 12, at 81 % at pH 5.8 and a volumetric hydrogen productivity rate (VHPR) of 0.55 L/(L.h) (Figure 3.1 d).

3.3. COD assays

Determination of both insoluble and soluble COD fractions from effluent samples provides a clear perspective of the metabolism occurring in the AFBR. Figure 3.1 (e-h) show COD concentrations of ammonia, hexoses, VFAs and biomass respectively. Biomass COD in the effluent fluctuated substantially throughout the time course with similar profiles observed for each of the other metabolites. Peaks in effluent biomass concentration were linked to optimal pH range and subsequently hydrogen productivities. The results for ammonia COD did not follow the profile as uniformly and seemed to oscillate every 3 – 4 days with the highest concentrations occurring over days 12 and 13 (530 and 584 mg/L respectively).

The VFA COD shows the collective concentration of all the fatty acids that could have been generated through fermentation, but primarily acetate, butyrate and propionate. If acetate were the primary VFA being generated then peaks in VFA COD should roughly correspond to peaks in hydrogen production; this is observed on day 11 with a COD of 813 mg/L and VHPR at 0.54 L/(L.h), with the hydrogen proportion of total gas production at 79 %.

The ammonia nitrogen consumption (Figure 3.1 i) shows a gradual decrease through the time course, with the greatest consumption occurring at bioreactor start-up. Assuming that ammonia loss was due to microbial consumption, then the rate of ammonia loss should correspond to microbial growth rates. Thus this plot can be used as an indicator of biomass growth and by showing fairly constant levels of ammonia nitrogen consumption implies steady-state maintenance of the bacterial population in the bioreactor during these intervals.

3.4. Cellulose feed profile

Figure 3.1 (l) shows the cumulative amount of steam-exploded wheat bran added to the AFBR. The arrows indicate time points, at which 200 g sucrose was added to stimulate microbial metabolic activity, reduce the pH level and promote biomass growth. For the first two weeks of operation, 300 g or 500 g wheat bran were added every 4 – 5 days. During the following weeks, 250 g was added once every 2 – 3 days. An average of 370 g wheat bran was added to the AFBR during the operational period with a cumulative total of 3.35 kg added over a period of 30 days.

3.5. Bacterial profiling

Molecular analyses conducted on the AFBR samples to establish profiles of the resident bacteria are depicted in figure 3.2 and 4.3. Isolated DNA from samples of day 1, 5, 9, 12, 17, 21 and 29 were subjected to PCR amplification targeting the 16S rDNA region. The agarose gel image (Figure 3.2) illustrates a successful reaction, with all samples being amplified. DGGE was subsequently conducted on samples of day 5, 12, 21, 29. Ideally, the selective conditions prevailing in the AFBR should reduce the diversity of bacterial species, allowing thermophilic, hydrogen-producing strains to proliferate. Sequencing results of the extracted fragments have as yet not been received.

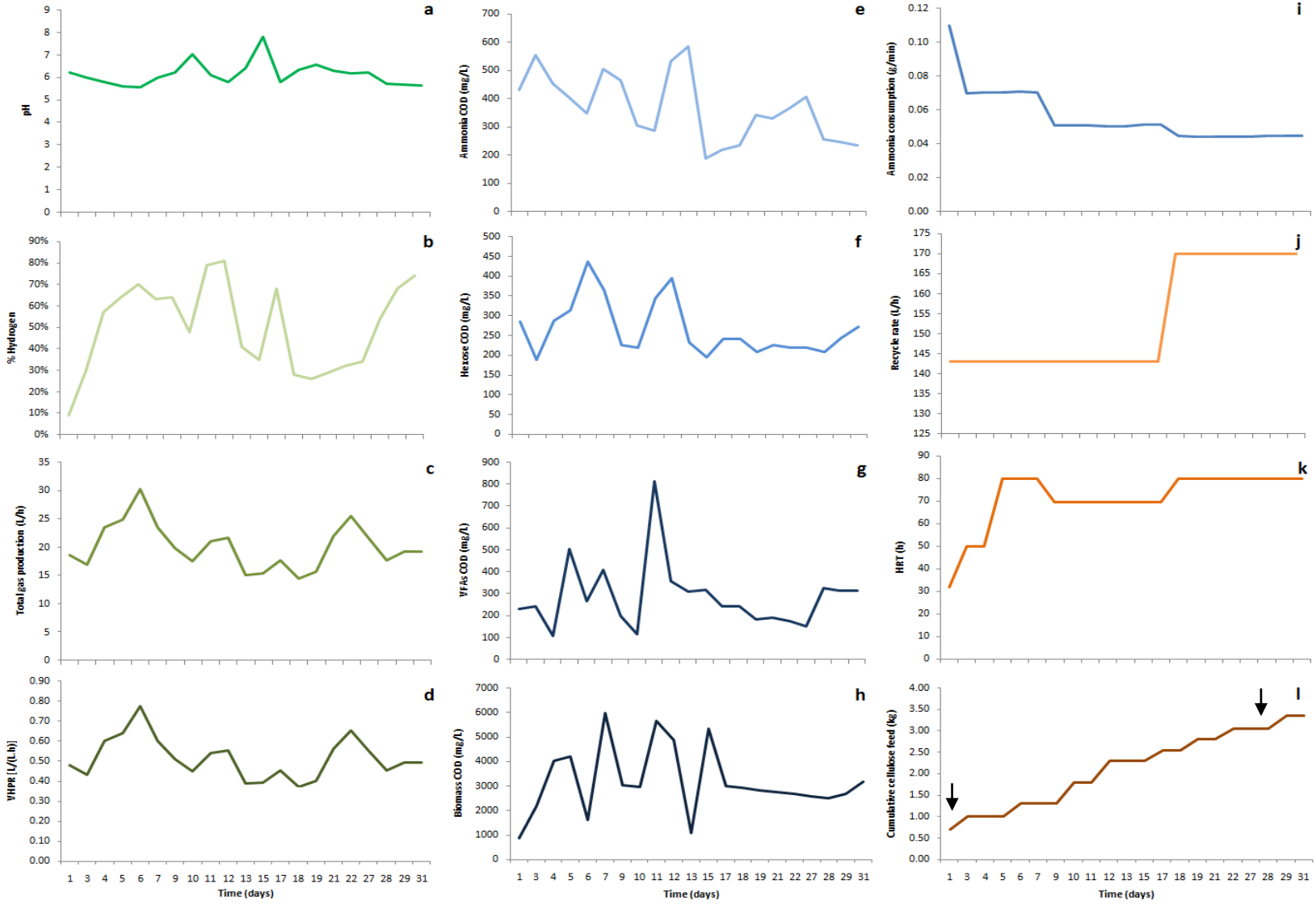


Figure 3.1. Time course profiles of parameter data obtained during bioreactor operation. (a) pH; (b) H_2 percentage of (c) total gas production; (d) VHPR; (e-h) COD concentrations for ammonia, hexoses, VFAs and biomass respectively; (i) ammonia consumption; (j) bioreactor recycle rate; (k) HRT; and (l) cumulative cellulose feed with arrows indicating sucrose addition.

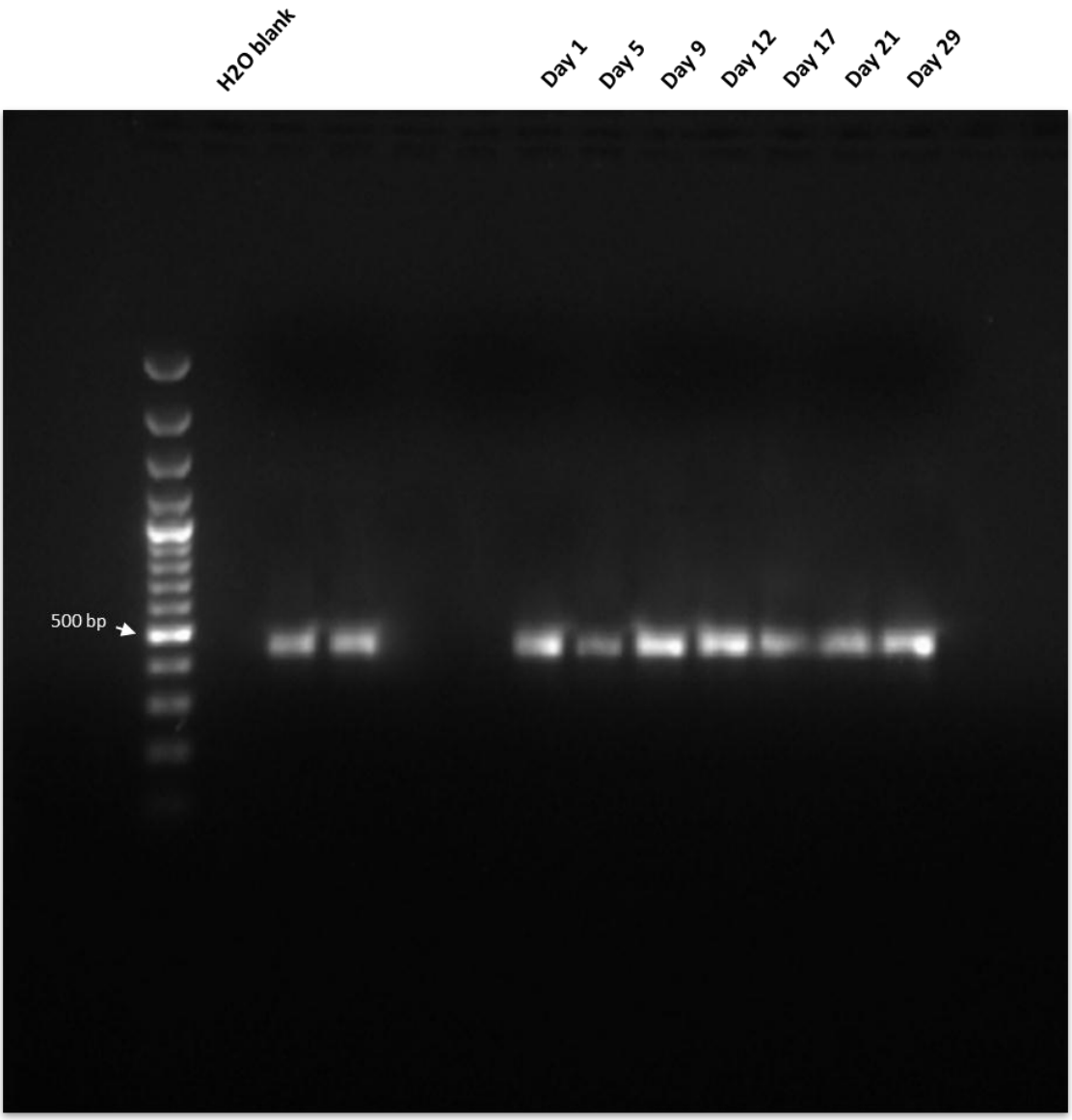


Figure 3.2. Agarose gel electrograph of successfully amplified 16S rDNA fragments (460 – 500 bp) obtained from DNA of samples on the respective days (labelled). Lane 1 showing a water blank indicating no contamination during the reaction. The unlabelled lanes containing amplified products were a confirmation of a previous amplification reaction.

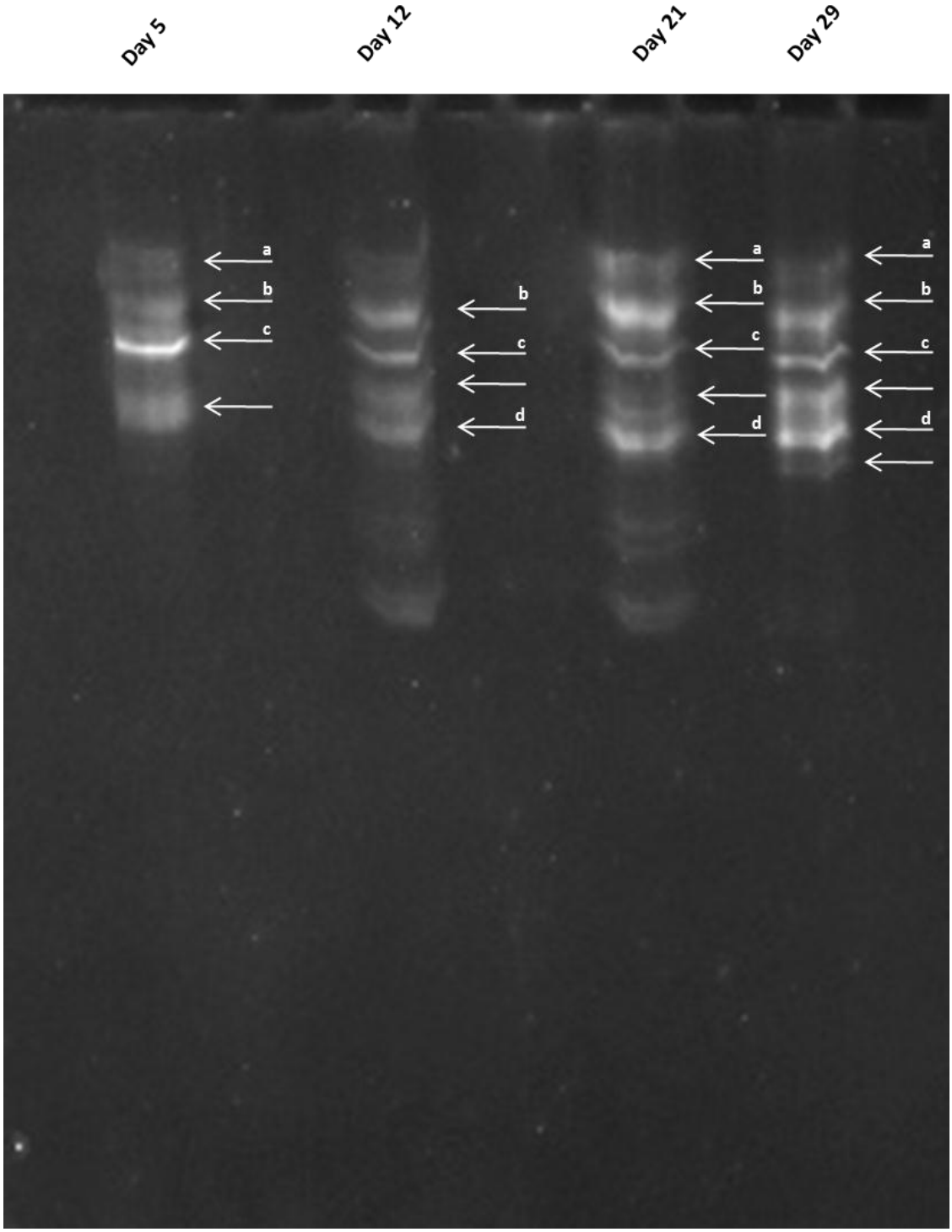


Figure 3.3. Polyacrylamide gel electrograph of a denaturing gradient electrophoresis performed on the PCR samples depicted. Arrows indicate distinct 16S rDNA fragments belonging to bacteria that were prevalent in the AFBR, while the letters (a-d) correspond to common bands on the respective sampling days. Day 5 shows a clearly dominant bacterial species. Faint bands are visible further down along the lanes of sample Day 12, Day 21 and Day 29, with a diminishing concentration in each, thus these were excluded from analysis.

4. DISCUSSION

While there is an abundance of research in the literature illustrating the impact of HRT on hydrogen productivity in continuous bioreactor systems, they are predominantly focussed on sucrose/glucose fermentation, whereas this study utilised cellulose as a carbohydrate source. By comparing the profiles of % H_2 and HRT (Figure 3.1 b and k respectively) it was observed that increasing the HRT from the initial 32 h to 80 h resulted in a correspondingly positive increase in H_2 percentage (peak at 70 %). High proportions of H_2 were maintained in the gas output for several days following (63 and 64 %) and a reduction in HRT to 70 h also yielded a reduction in hydrogen (48 %) although after approximately 48 hours, there was a considerable increase in hydrogen production (up to 81 %). This was the highest level achieved for the duration of AFBR operation, while the average overall hydrogen productivity was 50 %. Wu *et al*. (2008) and Zhang *et al*. (2006) demonstrated that lower HRTs (4 – 15 h) yielded hydrogen productivities of between 35 and 70 % using glucose/sucrose as carbohydrate substrates. While a study by Hawkes *et al.* (2008) utilising wheat-feed (cellulosic substrate) showed 70 % hydrogen productivity at an HRT of 15 h.

Although this study showed extremely high hydrogen productivities at HRTs of 70 and 80 h, those levels could not be maintained for more than a couple of hours and fluctuated significantly throughout the duration of the AFBR operation. Ideally however, lower HRTs (high dilution rates) are preferred in continuous AFBR systems to maximise hydrogen productivities and yields at an optimal steady-state of bacterial growth. This is feasible in reactors with high volumetric biomass densities and good settling properties that would prevent their washout (Lee *et al.*, 2006; Zhang *et al.*, 2008). This has not yet been shown in AFBRs using cellulosic materials as a substrate. Adjustments in HRT showed a small effect on overall VHPR (Figure 3.1 d) with HRT

of 80 h yielding the highest VHPRs: 0.64; 0.78 and 0.65 L/(L.h) respectively. An average VHPR of 0.51 L/(L.h) was attained over the period of operation.

The AFBR was run at two different recycle rates (143 and 170 L/h) and during both cases, hydrogen production levels fluctuated significantly. In AFBRs, recycle rate is critically important in maintaining fluidisation of the reactor bed, to allow maximum contact between bacterial biomass and cellulosic substrate, as well as recycling bacterial biomass overflow from the gas disengager through the fluid filter, and back into the main bioreactor column. Furthermore, the effect of recycling rate would homogenise influent nutrient Endo-medium being pumped in, throughout the reactor.

The effects of recycle rate in an AFBR on hydrogen production are poorly addressed in the scientific literature, although the agitation can be regarded as similar to the stirring effect present in CSTRs. As described earlier, CSTRs are the leading bioreactor systems for biohydrogen production studies. A study by Chou *et al.* (2008) on the effects of stirring rate in a CSTR showed enhanced hydrogen productivities when agitation was increased from 20 to 100 rpm although higher rates resulted in a gradual reduction of hydrogen production.

In AFBRs, high effluent recycle rates coupled with low HRTs facilitate increased hydrogen productivities and yields in bioreactor beds with high settling capability. In the case of this study the absence of a granulated bed, or one with elevated settling properties, could have explained the reason for the effluent recycle rate displaying no effect on hydrogen productivity.

To enhance initial microbial growth and metabolism and consequently promote accelerated start-up of the bioreactor system, sucrose was supplemented with the steam-exploded wheat-bran. This was performed again towards the end of the operational

period for the same purpose, however premature termination of the bioreactor system prevented further data acquisition. By assaying effluent hexose concentrations, the degradation of cellulose could be indirectly monitored. Figure 3.1 (f) shows the time course profile of effluent hexose COD (mg/L) and the greatest concentrations recorded (426 and 396 mg/L) corresponded to peaks of hydrogen productivities (70 and 81 % respectively).

The average effluent hexose COD concentration for the experimentation period was 264 mg/L and besides the two elevated instances at the hydrogen production peaks, there was comparatively little fluctuation in this value. This finding is peculiar considering that wheat-bran was added consistently every 2 - 4 days. A possible explanation is that the bacterial biomass metabolises the carbohydrate substrate rapidly enough that the entire system becomes starved of the carbon substrate until the next dispensation of wheat-bran. This would account for the relatively low concentrations of effluent hexose. However, the peaks during elevated hydrogen productivity would then signify a decreased utilisation of dissolved sugars within the bioreactor. This clearly cannot be the case. A molecular compositional analysis should be conducted on the wheat-bran to discern more precisely the amount of fermentable sugars present, prior to addition into the AFBR; thus allowing for the calculation of overall hexose conversion within the system, since the influent and effluent concentrations would be known.

Remembering the importance of ammonia nitrogen for bacterial growth, it is interesting to observe (Figure 3.1 e) the correlation to increased hydrogen production. The calculation of theoretical ammonia nitrogen consumption rate (g/min) (Figure 3.1 i) was highly perplexing. These data are theoretical in the sense that an assumption is being made that reductions in ammonia concentrations are due directly to microbial

consumption thereof and if this is true, then the data spread does not fit with the rest of the assays. This would have to be studied in further detail.

Looking at figure 3.1 (g) the effluent VFA COD share an approximately proportional relationship to the effluent hexose COD with peaks and troughs occurring concurrently. Elevated VFA concentrations during periods of high hydrogen productivities are expected, since the fermentative pathways allowing for biohydrogen production lead to the generation of various fatty acids, predominantly acetate and butyrate. In order to achieve sustained hydrogen productivity, the generation of VFAs (and the associated drop in pH) would need to compensate the pH increase effect from the influent nutrient medium being pumped into the system, such that the pH is maintained between 5.0 and 6.0.

As discussed initially, one of the fundamental factors driving hydrogen production is pH. There is no shortage of literature to corroborate this, with reviews detailing the numerous experiments conducted to determine optimum pH ranges (Davila-Vazquez *et al*., 2007; Wang and Wan, 2008; Hallenbeck and Ghosh, 2009). As mentioned above, the optimal pH range fermentative biohydrogen production exists between 5.0 and 6.0 however; the overall average over the duration of this study was 6.1. Nevertheless, during the periods where pH was maintained below 6.0, enhanced hydrogen production was evident. Comparing figure 3.1 (a) and (c) will illustrate this relationship. The study by Hawkes *et al*. (2008) showed sustained hydrogen production rates from wheat-feed fermentation when pH was set to 5.3. The possible ramification in the context of this study is therefore that the erratic hydrogen productivities observed were directly as a result of pH fluctuations. Of course, maintaining the pH would involve the optimisation of HRT and recycle rates together with maintaining addition of carbohydrate nutrition (wheat-bran) in a pseudo-continuous manner, to achieve a steady-state in resident

bacterial biomass growth. It is thus evident how the production of hydrogen is intricately dependent on the synergy of the AFBR parameters.

DGGE allows for DNA separation based on sequence differences and thereby producing a DNA fingerprint of the microbial species or strains present in a given sample. The DGGE performed in this study (Figure 3.3) showed increasing microbial diversity over the experimentation period, with the first sample possessing four prevalent strains with one predominating over the rest (day 5) right through to the sample of day 29 where six dominant strains are evident. Band fragments b and c were present throughout the sampling periods while band d became apparent from sampling day 12, this would be indicative of selective conditions prevailing in the bioreactor system. DNA corresponding to band A showed a decline from sampling day 5 to day 12, although the remaining sampling days showed a distinctive presence thereof, again indicating changes in the bioreactor, initially favouring that microbial species then selecting against it and once again selecting for it for the remaining sample periods. There may be a correlation between HRT and the DGGE results of the observed samples as day 5 and day 12 HRTs were 80 h and 70 h respectively while day 21 and 29 were both sampled at the same HRT (80 h).

It is interesting to note that day 12, which had the highest hydrogen productivity over the entire period, showed several strains prevailing with four predominating ones including bands b, c and d. Thus it can be assumed that these bands indicate a hydrogen-producing microbial consortium. Another point of attention is that the DGGE profiles of day 21 and 29 were very similar but the corresponding hydrogen productivities were drastically different, at 29 and 68 % respectively, despite possessing the strains associated with increased hydrogen productivity (b, c and d). Thus the evolutionary selection of the other microbial species during the last two sampling

periods is antagonistic to optimising acidogenic metabolism. Although the gel could have been electrophoresed for a longer period (possibly 5 h instead of 3 h) to obtain greater clarity and resolution between the DNA bands, the sequencing results will shed greater detail on these microbial profiles.

Preliminary assumptions can be made regarding the identities of the hydrogen-producing bacteria based on studies in the literature. The most likely candidate species would be *Clostridium* bacteria such as *C. thermocellum*, which has been shown to be cellulolytic and acidogenic (Levin *et al*., 2009; Lo *et al*., 2009). Other putative species include thermophilic bacteria such as *Thermoanaerobacterium thermosaccharolyticum*, *Caldicellulosiruptar saccharolyticum* and *Caldicellulosiruptor saccharolyticus* (Levin *et al*., 2009; Lo *et al*., 2009). The possibilities of syntrophic bacterial strains being present cannot be excluded since throughout the operational period there were levels of hydrogen production, and bacterial agglomerations may contain certain *Streptococcus* and *Pseudomonas* species that facilitate hydrogen production (Lo *et al*., 2009).

5. CONCLUSIONS

There is a general consensus in the literature that there are large practical hurdles that need to be overcome before biohydrogen production becomes industrially amenable. Primarily due to the metabolic constraint of 4 mol H_2 produced per mol glucose in dark fermentation. Therefore endeavours to innovate a biological process that is both practical and is able to extract 12 mol H_2 per mol glucose should be undertaken. According to Thauer *et al*. (1977) the thermodynamics of bacterial metabolism suggest that finding an organism capable of absolute conversion of carbohydrate substrates to hydrogen is impossible. Of course technology today is far more advanced than that of three decades ago and now, through human intervention, metabolic engineering is becoming increasingly popular as a method to enhance hydrogen production.

By modifying particular genes to which drive biohydrogen production (reviewed in Hallenbeck and Ghosh, 2009) a theoretical evolution of 12 mol H_2 is no longer an impossibility, but merely a research opportunity. These genetic modifications can result in increased biohydrogen-associated enzyme expression, or they can be gene knockouts of proteins involved in competing metabolic pathways. A step forward from this approach is the engineering of a completely new biohydrogen-producing pathway. In this scenario, *E. coli* would be used as the transgenic microorganism, containing the necessary genes to express a functional FeFe hydrogenase (highly active hydrogen-evolving enzyme native to fermentative Clostridia) (Hallenbeck and Ghosh, 2009). This is a relatively young field and tremendous research is still required to produce metabolically engineered bacteria that produce economically viable levels of hydrogen.

Hybrid two-stage systems are another alternative and have been discussed earlier as potential routes for enhanced biohydrogen production. Numerous research groups have

been collaborating to expedite technological development of industrially applicable bioreactor systems that are economically viable. Hyvolution is an example of a joint initiative between various academic institutions, research laboratories and government organisations. Projects such as this allow for an amalgamation of ideas with different research groups focussing on particular points of interest in the greater scheme of achieving an economically viable alternative energy source. The Bioprocess Research Lab of the University of the Witwatersrand is a member of the Hyvolution project and has made significant contributions in regards to AFBR systems for hydrogen production. This current study is part of the Hyvolution research task, and the results obtained will contribute to the larger body of knowledge surrounding AFBR bioprocesses and the utilisation of wheat-bran as a cellulosic carbohydrate substrate.

As a continuous / fed-batch bioprocess, the AFBR system utilising wheat-bran as a carbohydrate substrate proved to be unstable and highly variable with respect to hydrogen productivity. A peak hydrogen productivity of 81 % was obtained with an average of 50 % over the period of operation. Further evaluation of the effects of HRT would need to be done by possibly increasing the bioreactor running time to establish a granulated bed. The microbial profiles obtained need to be further analysed to discern conclusively the relationships being formed under the respective conditions (HRT, recycle rate, pH). Further ammonia nitrogen assays should be conducted to better explain the bizarre data spread (Figure 3.1 i - ammonia nitrogen consumption). As it stands hydrogen production was successful albeit erratic, and due to the integrated nature of the bioprocess conditions, it is difficult to isolate any single cause of these fluctuations. Further studies should look into examining the individual parameter effects under continuous conditions. Taken together the experimental objectives were

all achieved except for bed granulation, but as mentioned above this may require an extended operational period.

In the bigger picture of developing a sustainable alternative energy production system, the prospects of using a thermophilic AFBR system are not as optimistic. Through a personal communication with Prof. Vincent Gray (Bioprocess Research Lab, University of the Witwatersrand, Johannesburg) startling empirical evidence shows that if an AFBR operating at thermophilic temperatures, such as the one used in this study, were to be industrially up-scaled (41 L pre-pilot scale – 50 000 L industrial scale), there would be a net energy deficit. In that, the sum input of energy required to maintain a desired recycle rate through the entire system as well as heat the reactor from ambient temperature (25 °C) to a sustained thermophilic temperature (65 °C) would be greater than the output chemical biohydrogen energy (even at optimal hydrogen productivities and yields). This deficit in energy was revealed mathematically (calculations not shown) and as it stands, thermophilic anaerobic biohydrogen production in an AFBR is not practically viable, at commercial scales, as an alternative energy generating process. When considering integration into a two-stage process, the energy deficit would need to be addressed for practical and economical energy production. Table A1 in the appendix is a summary of calculations made to determine the energy balance.

By focusing on the improvement of hydrogen yields (above 4 mol/mol glucose) through metabolic engineering, this energy deficit will become an energy surplus and the real potential of biohydrogen generation may be realised. Substantial research thus awaits.

6. APPENDIX

Table A1. Energy balance for up-scaled thermophilic AFBR system model.

Energy inputs/outputs	Bioreactor energy balance (kWh)
Sensible heat convection	22
Radiant emittance	67
Heating of influent	8 380
Re-heating of recycled effluent	993
Mechanical energy	9 365
Total energy input	18 827
Optimum hydrogen energy output	15 345
Net useful energy production	**-3 482**

7. REFERENCES

Anderson J.M., and Ingram J.S.I. 1993. *Tropical soil biology and fertility – a handbook of methods*. 2nd Ed. 70-89. CAB UK.

Argun H., Kargi F., Kapdan I.K., Oztekin R. 2008. Biohydrogen production by dark fermentation of wheat powder solution**:** effects of C/N and C/P ratio on hydrogen yield and formation rate. *International Journal of Hydrogen Energy.* **33:** 1813 - 1819.

Benemann J.R. 1997. Feasibility analysis of photobiological hydrogen production. *International Journal of Hydrogen Energy*. **22:** 979 - 987.

Benemann J.R., Berenson J.A., Kaplan N.O., Kauren M.D. 1973. Hydrogen evolution by a chloroplast-ferredoxin-hydrogenase system. *Proceedings from the National Academy of Sciences USA*.**70:** 2317 - 2320.

Bisaillon A., Turcot J., Hallenbeck P.C. 2006. The effect of nutrient limitation on hydrogen production by batch cultures of Escherichia coli. *International Journal of Hydrogen Energy*. **31:** 1504 - 1508.

Bolton J.R. 1996. Solar photoproduction of hydrogen. Solar Energy **57:** 37 – 50.

Chen C.C. and Lin C.Y. 2003. Using sucrose as a substrate in an anaerobic hydrogen-producing reactor. *Advances in Environmental Research*. **7:** 695 - 699.

Cheong D.Y. and Hansen C.L. 2006. Bacterial stress enrichment enhances anaerobic hydrogen production in cattle manure sludge. *Appl Microbiol Biotechnol*. **72:** 635 - 643.

Chou C.H., Wang C.W., Huang C.C., Lay J.J. 2008. Pilot study of the influence of stirring and pH on anaerobes converting high-solid organic wastes to hydrogen. *International Journal of Hydrogen Energy.* **33:** 1550 – 1558.

Das D. 2009. Advances in biohydrogen production processes**:** An approach towards commercialization. *International Journal of Hydrogen Energy.* **34:** 7349 - 7357.

Das D. and Veziroglu T.N. 2001. Hydrogen production by biological processes: A literature survey. *International Journal of Hydrogen Energy.* **26:** 13 – 28.

Das D. and Veziroglu T.N. 2008. Advances in biological hydrogen production processes. *International Journal of Hydrogen Energy.* **33:** 6046 – 6057.

Davila-Vazquez G., Arriaga S., Alatriste-Mondragón F., de León-Rodríguez A., Rosales-Colunga L.M. *et al.* 2007. Fermentative biohydrogen production**:** Trends and perspectives. *Reviews in Environmental Science and Biotechnology*. **7:** 27 - 45.

de Mes T.Z.D., Stams A.J.M., Reith J.H., Zeeman G. 2003. Methane production by anaerobic digestion of wastewater and solid wastes. In**:** Reith J.H., Wijffels R.H., Barten H. (eds) *Bio-methane & biohydrogen**:** status and perspectives of biological methane and hydrogen production*. 58 - 102. Dutch Biological Hydrogen Foundation, Petten, The Netherlands.

Endo G., Noike T., Matsumoto J. 1982. Characteristics of cellulose and glucose decomposition in acidogenic phase of anaerobic digestion (in Japanese). *Proceedings of the Japanese Society of Civil Engineering.* **325:** 61 - 68.

Fascetti E. and Todini O. 1995. Rhodobacter sphaeroides RV cultivation and hydrogen production in a one- and two-stage chemostat. *Applied Microbiology and Biotechnology*. **22:** 300 - 305.

Fascetti E., D' Addario E., Todini O., Robertiello A. 1998. Photosynthetic hydrogen evolution with volatile organic acids derived from the fermentation of source selected municipal solid wastes. *International Journal of Hydrogen Energy*. **23:** 753 - 760.

Fedorov A.S., Tsygankov A.A., Rao K.K., Hall D.O. 1998. Hydrogen photo-production by Rhodobacter sphaeroides immobilised on polyurethane foam. *Biotechnology Letters*. **20:** 1007 - 1009.

Francou N. and Vignais P.M. 1984. Hydrogen production by Rhodopseudomonas capsulata cells entrapped in carrageenan beads. *Biotechnology Letters.* **6:** 639 644.

Gaffron H. and Rubin J. 1992. Fermentative and photochemical production of hydrogen by algae. *Journal of General Physiology*. **26:** 219 - 240.

Gavala H.N., Skiadas I.V., Ahring B.K. 2006. Biological hydrogen production in suspended and attached growth anaerobic reactor systems. *International Journal of Hydrogen Energy*. **31:** 1164 - 1175.

Gottschalk G. 1986. Bacterial metabolism. 2nd Ed. Springer

Greenbaum E. 1990. Hydrogen production by photosynthetic water splitting. In**:** Veziroglu T.N. and Takashashi P.K. (eds). *Hydrogen energy progress VIII*. Proceedings 8th WHEC, Hawaii. 743 - 754 Pergamon Press, New York.

Hallenbeck P.C. 2005. Fundamentals of the fermentative production of hydrogen. *Water Science and Technology*. **52:** 21 - 29.

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

Hallenbeck P.C. and Ghosh D. 2009. Advances in fermentative biohydrogen production**:** the way forward? *Trends in Biotechnology*. **27:** 287 - 297.

Hawkes F.R., Forsey H., Premier G.C., Dinsdale R.M., Hawkes D.L. *et al*. 2008. Fermentative production of hydrogen from a wheat flour industry co-product. *Bioresource Technology* **99:** 5020 – 5029.

Hu B. and Chen S.L. 2007. Pretreatment of methanogenic granules for immobilized hydrogen fermentation. *International Journal of Hydrogen Energy*. **32:** 3266 - 3273.

Ishikawa M., Yamamura S., Tamkamura Y., Sode K., Tamiya E. *et al*. 2006. Development of a compact high-density microbial hydrogen reactor for portable bio-fuel cell system. *International Journal of Hydrogen Energy*. **31:** 1484 - 1489.

Kim J.S., Ito K., Takahashi H. 1981. Production of molecular hydrogen by Rhodopseudomonas sp. *Journal of Fermentation and Technology.* **59:** 185 - 190.

Kim M.S., Lee T.J., Yoon Y.S., Lee I.G., Moon K.W. 2001. Hydrogen production from food processing wastewater and sewage sludge by anaerobic dark fermentation combined with photofermentation. In**:** Miyake J., Matsunaga T., Pietro A.S. (eds). *Biohydrogen II*. 263 - 72. Elsevier, Amsterdam.

Kleerebezem R. and van Loosdrecht M.C.M. 2007. Mixed culture biotechnology for bioenergy production. *Current Opinion in Biotechnology*. **18:** 207- 212.

Kotsopoulos T.A., Zeng R.J., Angelidaki I. 2006. Biohydrogen production in granular up-flow anaerobic sludge blanket (UASB) reactors with mixed cultures under hyper-thermophilic temperature (70 °C). *Biotechnology and Bioengineering*. **94:** 296 - 302.

Lambert G.R. and Smith G.D. 1980. Hydrogen metabolism by filamentous cyanobacteria. *Archives in Biochemistry and Biophysics.* **205:** 36 - 50.

LaPara T.M., Alleman J.E., Pope P.G. 2000. Miniaturised closed reflux, colorimetric method for the determination of chemical oxygen demand. *Waste Management.* **20:** 295 - 298.

Lee C.M., Chen P.C., Wang C.C., Tung Y.C. 2002. Photohydrogen production using purple non-sulfur bacteria with hydrogen fermentation reactor effluent. *International Journal of Hydrogen Energy*. **27:** 1308 - 1314.

Lee K.S., Lin P.J., Chang J.S. 2006. Temperature effects on biohydrogen production in a granular sludge bed induced by activated carbon carriers. *International Journal of Hydrogen Energy*. **31:** 465 - 472.

Lee K.S., Lin P.J., Fangchiang K., Chang J.S. 2007. Continuous hydrogen production by anaerobic mixed microflora using a hollow-fiber microfiltration membrane bioreactor. *International Journal of Hydrogen Energy*. **32:** 950 - 957.

Lee K.S., Lo Y.S., Lo Y.C., Lin P.J., Chang J.S. 2003. H_2 production with anaerobic sludge using activated-carbon support packed-bed bioreactors. *Biotechnology Letters.* **25:** 133 - 138.

Levin D.B., Carere C.R., Cicek N., Sparling R. 2009. Challenges for biohydrogen production via direct lignocellulose fermentation. *International Journal of Hydrogen Energy.* **34:** 7390 - 7403.

Levin D.B., Pitt L., Love M. 2004. Biohydrogen production**:** prospects and limitations to practical application. *International Journal of Hydrogen Energy*. **29:** 173 - 185.

Li C.L. and Fang H.H.P. 2007. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. *Critical Reviews in Environmental Science and Technology*. **37:** 1 - 39.

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

Lin C.Y. and Lay C.H. 2005. A nutrient formulation for fermentative hydrogen production using anaerobic sewage sludge microflora. *International Journal of Hydrogen Energy*. **30:** 285 - 292.

Liu H., Got S., Logan B.E. 2005. Electrochemically assisted microbial production of hydrogen from acetate. *Environmental Science and Technology*. **39:** 4317 - 4320.

Lo Y.C., Saratale G.D., Chen W.M., Bai M.D., Chang J.S. 2009. Isolation of cellulose-hydrolytic bacteria and applications of the cellulolytic enzymes for cellulosic biohydrogen production. *Enzyme & Microbial Technology.* **44:** 417 - 425.

Lodhi M.A.K. 1987. Hydrogen production from renewable sources of energy. *International Journal of Hydrogen Energy*. **12:** 461 - 468.

McInerney M.J., Rohlin L., Mouttaki H., Kim M., Rebecca S. *et al*. 2007. The genome of Syntrophus aciditrophicus**:** life at the thermodynamic limit of microbial growth. *Proceedings of the National Academy of Science USA*. **104:** 7600 - 7605.

Miyake J. and Kawamura S. 1987. Efficiency of light energy conversion to hydrogen by photosynthetic bacteria Rhodobacter sphaeroides. *International Journal of Hydrogen Energy*. **12:** 147 - 149.

Mohan S.V., Babu V.L., Sarma P.N. 2008. Effect of various pretreatment methods on anaerobic mixed microflora to enhance biohydrogen production utilizing dairy wastewater as substrate. *Bioresource Technology*. **99:** 59 - 67.

Morris D.L. 1948. Determination of carbohydrate in biological fluids. *Science*. **107:** 254.

Mu Y., Yu H.Q., Wang Y. 2006. The role of pH in the fermentative H2 production from an acidogenic granule-based reactor. *Chemosphere*. **64:** 350 - 358.

Nath K. and Das D. 2003.Hydrogen from biomass. *Current Science*. **85:** 265 - 271.

Nath K. and Das D. 2005. Hydrogen production by Rhodobacter sphaeroides strain O.U. 001 using spent media of Enterobacter cloacae strain DM11. *Applied Microbiology and Biotechnology*. **68:** 533 - 541.

Oh Y.K., Kim S.H., Kim M.S., Park S. 2004. Thermophilic biohydrogen production from glucose with trickling biofilter. *Biotechnology and Bioengineering*. **88:** 690 - 698.

O-Thong S., Prasertsan P., Intrasungkha N., Dhamwichukorn S., Birkeland N.K. 2008. Optimization of simultaneous thermophilic fermentative hydrogen production and COD reduction from palm oil mill effluent by Thermoanaerobacterium-rich sludge. *International Journal of Hydrogen Energy*. **33:** 1221 - 1231.

Ramachandran R. and Menon R.K. 1998. An overview of industrial uses of hydrogen. . *International Journal of Hydrogen Energy*. **23:** 593 - 598.

Ren N., Lia J., Lib B., Wanga Y., Liu S. 2006. Biohydrogen production from molasses by anaerobic fermentation with a pilot-scale bioreactor system. *International Journal of Hydrogen Energy*. **31:** 2147 - 2157.

Rosen M.A. and Scott D.S. 1998. Comparative efficiency assessments for a range of hydrogen production processes. *International Journal of Hydrogen Energy*. **23:** 653 - 659.

Salerno M.B., Park W., Zuo Y., Logan B.E. 2006. Inhibition of biohydrogen production by ammonia. *Water Research*. **40:** 1167 - 1172.

Sarker S., Pandey K.D., Kashyap A.K. 1992. Hydrogen photoproduction by filamentous non-heterocystous cyanobacterium Pleatonema boryanna and simultaneous release of ammonia. *International Journal of Hydrogen Energy*. **17:** 689 - 694.

Sasikala K., Ramana C.V., Rao P.R., Kovacs K.L. 1993. Anoxygenic phototrophic bacteria**:** physiology and advances in hydrogen technology. *Advances in Applied Microbiology*. **10:** 211 - 215.

Schotz F. and Schroder U. 2003. Bacterial batteries. *Nature Biotechnology*. **21:** 3 - 4.

Smith G.D., Ewart G.D., Tucker W. 1992. Hydrogen production by cyanobacteria. *International Journal of Hydrogen Energy*. **17:** 695 - 698.

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

Solorzano L. 1969. Determination of ammonia in natural waters by the phenolhypochlorite method. *Limnology and Oceanography*. **14:** 799.

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

Suzuki Y. 1982. On hydrogen as fuel gas. *International Journal of Hydrogen Energy*. **7:** 227 - 230.

Thauer R.K, Jungermann K., Decke K. 1977. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriological Reviews*. **41:** 100 - 180.

Ting C.H., Lin K.R., Lee D.J., Tay J.H. 2004. Production of hydrogen and methane from wastewater sludge using anaerobic fermentation. *Water Science and Technology*. **50:** 223 - 228.

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

Vincenzini M., Materassi R., Sili C., Florenzano G. 1986. Hydrogen production by immobilized cells III**:** prolonged and stable H2 photoevolution by Rhodopseudomonas palaustris in light-darkcycles. *International Journal of Hydrogen Energy*. **11:** 623 - 626.

Vincezini M., Materassi R., Tredici M.R., Florenzano G.1982. Hydrogen production by immobilized cells I: Light dependent dissimilation of organic substances by Rhodospeudomonas palustris *International Journal of Hydrogen Energy*.**7:** 231 - 236.

Wang J.L. and Wan W. 2008. Comparison of different pretreatment methods for enriching hydrogen-producing cultures from digested sludge. *International Journal of Hydrogen Energy*. **33:** 2934 - 2941.

Wang J.L. and Wan W. 2009. Factors influencing fermentative hydrogen production**:** A review. *International Journal of Hydrogen Energy.* **34:** 799 - 811.

Wu S.Y., Hung C.H., Lin C.Y., Lin P.J., Lee K.S. *et al*. 2008. HRT-dependent hydrogen production and bacterial community structure of mixed anaerobic microflora in suspended, granular and immobilized sludge systems using glucose as the carbon substrate. *International Journal of Hydrogen Energy.* **33:** 1542 - 1549.

Zhang Y.F., Liu G.Z., Shen J.Q. 2005. Hydrogen production in batch culture of mixed bacteria with sucrose under different iron concentrations. *International Journal of Hydrogen Energy.* **30:** 855 - 860.

Zhang Z.P., Show K.Y., Tay J.H., Liang D.T., Lee D.J. 2008. Biohydrogen production with anaerobic fluidized bed reactors - A comparison of biofilm-based and granule-based systems. *Process Biochemistry*. **33:** 1559 - 1564.

Zhang Z.P., Show K.Y., Tay J.H., Liang D.T., Lee D.J. *et al*. 2006. Effect of hydraulic retention time on biohydrogen production and anaerobic microbial community. *Process Biochemistry*. **41:** 2118 - 2123.

Zhu H.G. and Beland M. 2006. Evaluation of alternative methods of preparing hydrogen producing seeds from digested wastewater sludge. *International Journal of Hydrogen Energy*. **31:** 1980 - 1988.

Zurrer H. and Bachofen R. 1979. Hydrogen production by the photosynthetic bacterium, Rhodospirillum rubrum. *Applied Environmental Microbiology*. **37:** 789 - 793.