MODELLING AND OPTIMIZATION OF MICROBIAL PRODUCTION OF HYDROGEN ON AGRO-MUNICIPAL WASTES

A dissertation submitted to the College of Agriculture, Engineering and Science, in fulfillment of the academic requirements for the degree of

Master of Science

By

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STUDENT DECLARATION

Modelling and optimization of microbial production of hydrogen on agro-municipal wastes

I **Patrick Thabang Sekoai** declares that:

1. The research reported in this dissertation, except where otherwise indicated; is the result of my own endeavours in the Discipline of Microbiology, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg.
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I hereby declare that I acted as a supervisor for this MSc student:

Student's Full Name: Patrick Thabang Sekoai

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Thesis Title: Modelling and optimization of microbial production of hydrogen on agro-municipal wastes

Regular consultation took place between the student and me throughout the course of this research. I advised the student to the best of my ability and approved the final document for submission to the College of Agriculture, Engineering and Science Higher Degrees Office for examination by the University appointed Examiners.

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DECLARATION 2- PUBLICATIONS

This thesis involves a compilation of published work. The first author (student) contributed in experimental work, data collection and manuscript preparation, guided by the second author (supervisor).

Publication 1:

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Publication 2:

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Publication 3:

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CONFERENCE CONTRIBUTIONS

I. **Sekoai, P.T.** Potential of using Organic Fraction of Solid Municipal Waste (OFSMW) for biohydrogen production in South Africa. Postgraduate Energy Conference (EPC) 2013. iThemba Labs. Cape Town, Somerset West, 11-14 August 2013, Oral presentation.

ABSTRACT

The indiscriminate use of fossil fuels has led to global problems of greenhouse gas emissions, environmental degradation and energy security. Developments of alternative and sustainable energy resources have assumed paramount importance over the past decades to curb these challenges. Biohydrogen is emerging as an alternative renewable source of energy and has received considerable attention in recent years due to its social, economic and environmental benefits. It can be generated by dark fermentation on Organic Fraction of Solid Municipal Waste (OFSMW). These OFSMW exist abundantly and poses disposal challenges. This study models and optimizes the production of biohydrogen on a mixture of agro-municipal wastes; it examines a semi-pilot scale production on these substrates and the feasibility of generating bioelectricity from the process effluents and reviews the prospect of enhancing fermentative biohydrogen development using miniaturized parallel bioreactors.

The fermentation process of biohydrogen production on agro-municipal wastes was modelled and optimized using a two-stage design. A mixture design was used for determination of optimum proportions of co-substrates of Bean Husk (BH), Corn Stalk (CS) and OFSMW for biohydrogen production. The effects of operational setpoint parameters of substrate concentration, pH, temperature and Hydraulic Retention Time (HRT) on hydrogen response using the mixed substrates were modelled and optimized using box-behnken design. The optimized mixtures were in the ratio of OFSMW: BH: CS = 30:0:0 and OFSMW: BH: CS = 15:15:0 with yields of 56.47 ml H_2/g TVS and 41.16 ml H_2/g TVS respectively. Optimization on physico-chemical parameters using the improved substrate suggested optimal setpoints of 40.45 g/l, 7.9, 30.29 ^oC and 86.28 h for substrate concentration, pH, temperature and HRT respectively and hydrogen yield of 57.73 ml H_2/g TVS. The quadratic polynomial models from the mixture and box-behnken design had a coefficient of determination (R^2) of 0.94 and 0.79 respectively, suggesting that the models were adequate to navigate the optimization space.

The feasibility of a large-scale biohydrogen fermentation process was studied using the optimized operational setpoints. A semi-pilot scale biohydrogen fermentation process was carried out in 10 L bioreactor and the potential of generating bioelectricity from the process effluents was further assessed using a two-chambered Microbial Fuel Cell (MFC) process. The maximum hydrogen fraction of 46.7% and hydrogen yield of 246.93 ml H_2/g TVS were

obtained from the semi-pilot process. The maximum electrical power and current densities of 0.21 W/m^2 and 0.74 A/m^2 respectively were recorded at 500 Ω and the chemical oxygen demand (COD) removal efficiency of 50.1% was achieved from the MFC process.

This study has highlighted the feasibility of applying agricultural and municipal wastes for large-scale microbial production of hydrogen, with a simultaneous generation of bioelectricity from the process effluents. Furthermore, the potential of generating an economical feasible biohydrogen production process from these waste materials was demonstrated in this work.

Keywords: Biohydrogen production, Organic Fraction of Solid Municipal Waste (OFSMW), Modelling and optimization, Fermentation process, Renewable energy, Bioenergy

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Note: This thesis involves a compilation of published chapters whereby each chapter is presented as an individual entity according to each journal's regulations. Hence there is repetition between chapters.

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LIST OF ABBREVIATIONS

BH…..	Bean Husk
CD…..	Current Density
CH_4…..	Methane
CO_2…..	Carbon Dioxide
COD…..	Chemical Oxygen Demand
CS…..	Corn Stalk
DEA…..	Department of Environmental Affairs
DGGE…..	Denaturing Gradient Gel Electrophoresis
DNA…..	Deoxyribonucleic Acid
DOE…..	United States Department of Energy
DWAF…..	Department of Water Affairs and Forestry
EIA…..	United States Energy Information Administration
h…..	hour
H_2…..	Hydrogen
HRT…..	Hydraulic Retention Time
I…..	Current
IEA…..	International Energy Agency
IPCC…..	Intergovernmental Panel on Climate Change
MFC…..	Microbial Fuel Cell
OFSMW…..	Organic Fraction of Solid Municipal Waste
OLR…..	Organic Loading Rate
P…..	Electrical Power
PCR…..	Polymerase Chain Reaction
PD…..	Power Density
rpm…..	revolutions per minutes
TVS…..	Total Volatile Solids
V…..	Voltage
VFA…..	Volatile Fatty Acids
WHO…..	World Health Organization

CHAPTER 1

General introduction

1.1. The need for alternative energy

The reliance on fossil fuels has led to catastrophic climate change and environmental concerns (Davila-Vazquez *et al.*, 2008) and with alarming impacts on human health. These range from direct effects such as heat stress and flooding, to indirect influences including changes in disease transmission and malnutrition in response to increased competition for crop and water resources (VijayaVenkataRaman *et al.*, 2012). Thus, the World Health Organization (WHO) estimates that millions of people die each year from the side effects of climate change (WHO, 2008). In addition, changing weather patterns are expected to alter the geographical distribution of insect vectors that spread infectious diseases. The energy demands are projected to increase exponentially over the next three decades as a result of economic growth from developing nations and population throughout the world (Zurawski *et al.*, 2005).

The global trend in oil discovery and production is shown in Figures 1.1 and 1.2 respectively. It is apparent that fossil fuel reserves have reached their peak production. Based on the peak oil theory, a region's natural reserves reaches a peak production when half of the recoverable resources have been consumed (Bentley, 2002). Oil reserves have been declining since the 1960's as shown in Figure 1.1 due to high demand and overuse in developed countries. Moreover, it's envisioned that the global oil supply will be less than 10 Gb/a (Gigabarrels per annum) in 2015. This data presents a looming energy crisis considering the fact that the current global energy consumption is heading towards 1 Gb/a (BP, 2013). Fossil fuel reserves are geographically unevenly distributed in the world and are being depleted (Ruying, 2007). The main supplier of global conventional oil is the Middle East, and has an estimated oil capacity of 730 billion barrels (bnbl). This region controls a huge percentage of energy reserves (EIA, 2005). The United States Geological Survey (USGS) showed that more than 50% of oil is produced by South Arabia, Iran, Iraq, Kuwait, United Arab Emirates and Libya (USGS, 2007). In 2006, the Middle East supplied 22% of oil to the United States, 36% to Europe, 40% to China, 60% to India, and 80% to Japan and South Korea (EIA, 2005).

The global oil supply is therefore at risk because the total conventional oil production from all the countries in the world except for Middle East has reached a peak production (Campbell, 1991). However there are uncertainties about the reliance on the Middle East due to the sustained and increasingly worse political turmoil in this region. The region is faced with challenges of unstable governments, increasing terrorists' activities against oil reserves, and lack of economic stability (MECAD, 2013). This poses a threat to global energy security. Furthermore, future projections show that oil reserves in the Middle East will soon reach a peak production due to high supply. A study conducted by the United Kingdom Energy Research Council in 2009 predicted a peak in oil production in this region occurring before 2020 (Sorrell *et al.*, 2009).

The global greenhouse gas emission for 2008 is shown in Figure 1.3. The main greenhouse gases are carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and halocarbons (Stern, 2008). CO_2 is most abundant anthropogenic greenhouse gas in the atmosphere and the main contributor to climate change (Stern, 2008). It is derived from the combustion of fossil fuels and 62% are released into the atmosphere (Figure 1.3). Data from the International Energy Agency (IEA) estimated that 30 billion tonnes of CO_2 were emitted from fossil fuels in 2008 and this value has doubled since 1970 (IEA, 2011). Studies show that CO_2 levels have increased to 390 ppm since 2007, which is an average increase of 3.30 ppm per year during the last 6 years (Tans and Keeling, 2011). It has been projected that if no action is taken, the concentration of CO_2 in the atmosphere could increase up to 560 ppm by 2035 with a consequent temperature rise that could exceed 5 °C (Stern, 2008).

The World Bank (2013) has indicated that the ongoing global warming could leads millions of people to poverty. Studies show that Africa and Asia will suffer severely from the effects of climate change. For example, it is predicted that 40% of land used for maize production in sub-Saharan Africa will not be arable by 2030 due to devastating environmental effects of heat, drought and floods. Asia will experience more intense cyclones and a rise in sea levels (World Bank, 2013). A review by Schmidhuber and Tubiello (2007) showed that climate change will have huge drawbacks on food security. In semi-arid areas, droughts will dramatically reduce crop yields and livestock mortality (Cooper *et al.*, 2008). Most of this land is in sub-Saharan Africa and parts of South Asia. In dry regions, climate models predicted an increase in evapotranspiration and lower soil moisture levels (Cooper *et al.*, 2008). Some cultivated areas

may become unsuitable for farming and some tropical grassland may become increasingly arid. In Mediterranean regions, there are high risks of flooding including the possibility of increased coastal storms as results of temperature rise (Rosenzweig *et al.*, 2002). Hence, climate change is highlighted as a fundamental threat to global economic development and prosperity (World Bank, 2013).

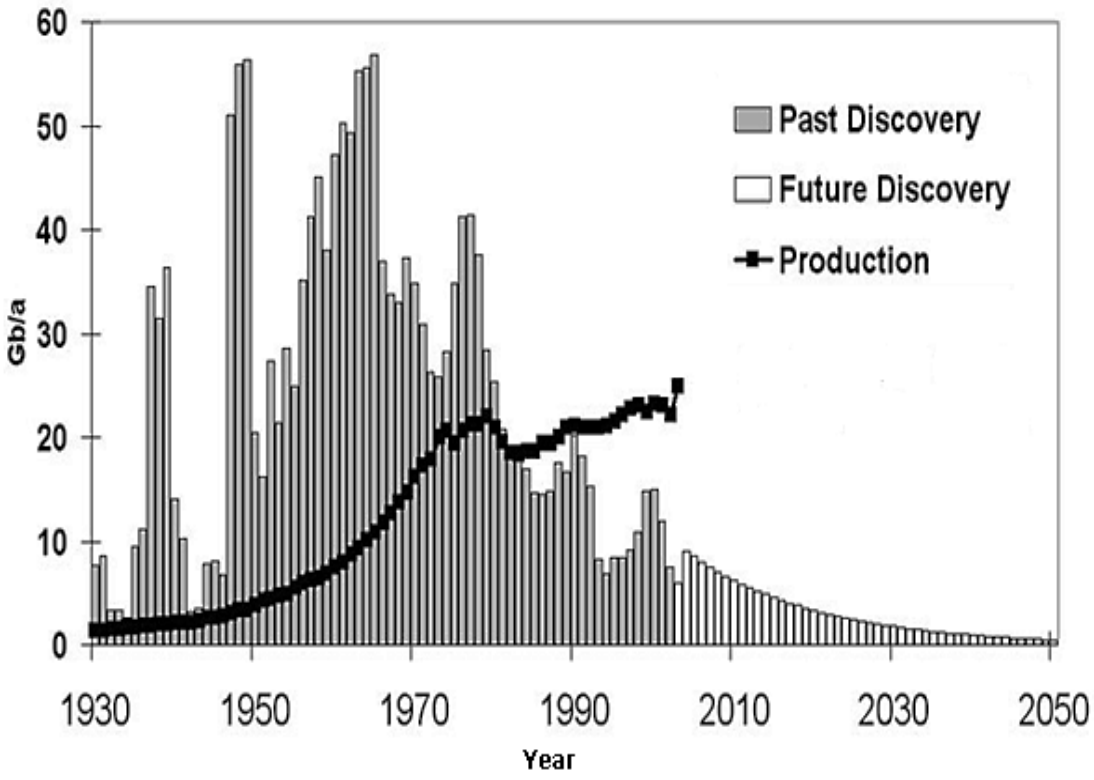

Figure 1.1: The global oil discovery and production (Longwell, 2002).

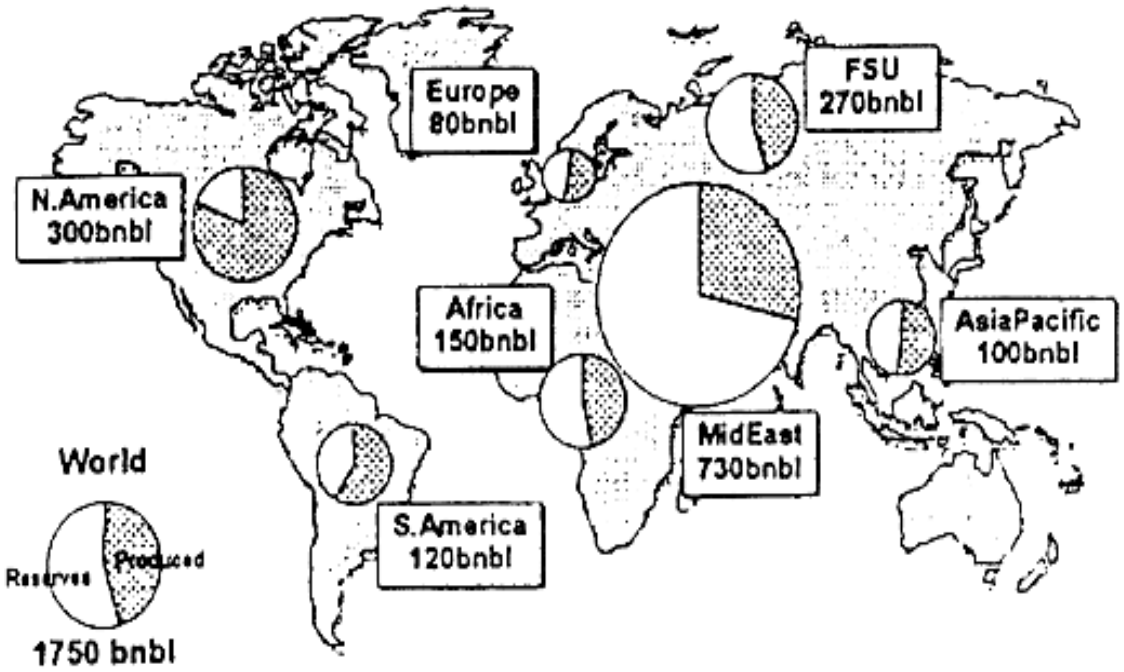

Figure 1.2: Global conventional oil distribution: shows the world's conventional oil that have been consumed (dark region), and the currently discovered reserves (unshaded region). (BP, 1999).

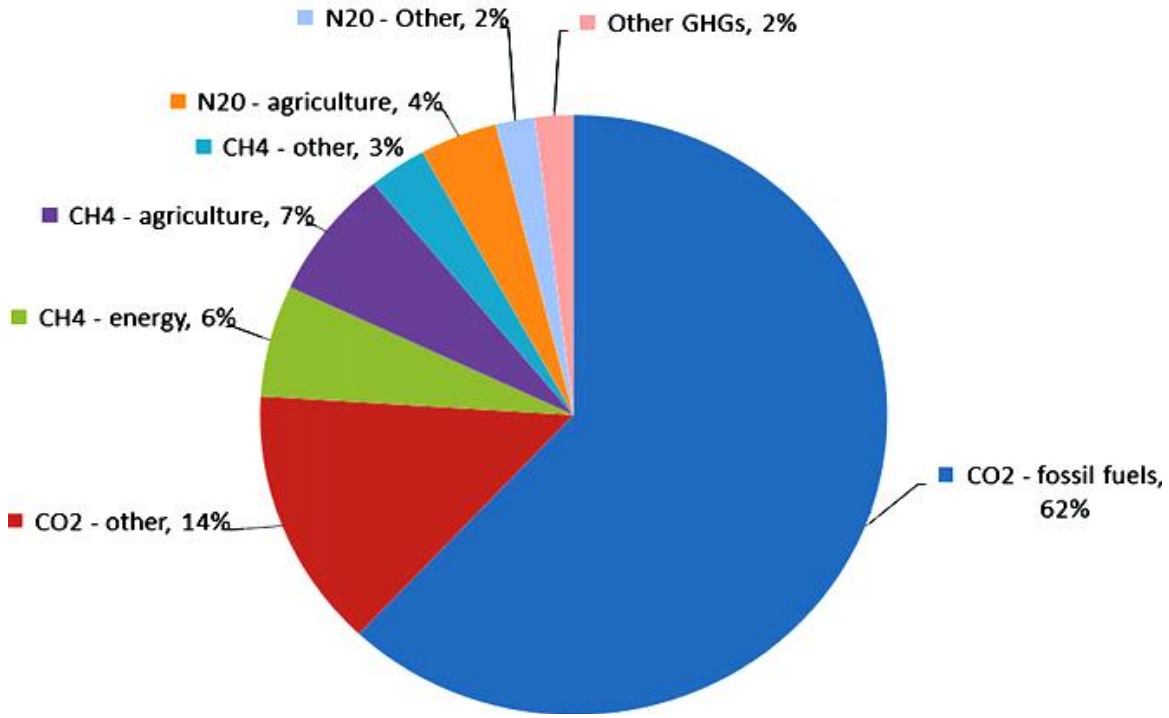

Figure 1.3: Global greenhouse gas emissions for 2008 (EIA, 2011).

Energy security is therefore regarded as a huge challenge in the 21st century together with anthropogenic climate change (McCartney *et al.,* 2008), the current employed energy systems will not be able to cope with future energy demands. The United States Energy Information Administration (EIA) showed that more than 80% of global primary energy is derived from fossil fuels with oil accounting for 32.8%, coal for 27.2% and natural gas for 20.9% (EIA, 2011). Nonetheless fossil fuels are being depleted and their production is closely linked to environmental degradation that threatens human health and quality of life, and affects ecological balance and biological diversity. Thus, it is apparent that if the rapidly increasing global energy needs are to be met without irreparable environmental damage, there will have to be a worldwide drive to exploit energy systems that should not endanger the life of current and future generations and should not exceed the carrying capacity of ecosystems (Asif and Muneer, 2007). In addition, the world population is projected to reach 8.3 billion by 2030, which means an additional 1.3 billion people will need energy (BP, 2013). An energy crisis is looming and it is speculated that by 2050 energy demands will outstrip supply (Holmes and Jones, 2003).

Recent analysis of the transformations required in the global energy system suggests that renewable based technologies will play a significant role in the global energy supply in the next decades (Dornburg *et al.,* 2010). Currently they are only contributing 13.5% of global energy supply (Asif and Muneer, 2007). This underscores a need to fast-track the development of alternative energy resources in order to meet high global energy demands and reduce carbon dioxide emissions.

1.2. Hydrogen as a potential energy source

Hydrogen is considered as one of the most promising energy carriers, because of its high efficiency of conversion to usable power, non-polluting oxidation products, and high gravimetric energy (Cheng and Liu, 2011). These advantages make it an attractive candidate to reduce reliance on conventional fossil fuels (Elsharnouby *et al.,* 2013). It has been reported that 50 million tonnes of hydrogen are traded annually worldwide with a growth rate of approximately 10% (Winter, 2005). According to the United States Department of Energy (DOE), hydrogen contribution to total energy market will be 6-10% by 2025 (DOE, 2004). Most developed countries in the world have therefore recognized the pivotal role that hydrogen may contribute in the future and thus experts are advocating the concept of a hydrogen economy (Turner, 2004). In

light of this development, the need of sustainable and sufficient supply of hydrogen is inevitably in great demand (Wu *et al.,* 2006). Fermentative hydrogen process development has gained a tremendous impetus and governmental support in more than 30 countries (Meher Kotay and Das, 2008). Currently, there are more than 400 projects globally that focus primarily on the implementation of hydrogen as alternative energy source. These activities are part of a global effort to increase energy security, environmental protection, and economic prosperity by commercialization of hydrogen (EIA, 2011).

The projected trends in global carbon dioxide emissions and hydrogen infrastructure development from 1900 to 2100 are shown in Figure 1.4. Carbon emissions from energy use and industrial sources are estimated to increase from 6:2 GtC (gigatonne of carbon = 10^9 of carbon) in 1990 to 14:2 GtC in 2100. Meanwhile, hydrogen producing technologies are expected to increase significantly, from 6% in 2020 to 50% in 2050 due to increasing energy demands. During this period, hydrogen infrastructures (B1-H_2) will develop and become progressively more important in decarbonizing the energy system. Emissions peak around 10:5 GtC in 2040 will decrease in 2100 (5:7 GtC), when hydrogen technologies are implemented on industrial scale (Figure 1.4).

Hydrogen is produced using various processes such as electrochemical, thermochemical, photochemical, photocatalytic, and photoelectrochemical processes (Momirlan and Veziroglu, 2002). However, these processes are energy intensive and expensive. They also do not accomplish the dual goals of waste reduction and energy conservation (Han and Shin, 2004). One attractive route for commercial production of hydrogen is to use biological processes. Biological hydrogen production process is one of the more environmental friendly and less energy intensive methods, thereby being more competitive to conventional hydrogen production methods such as thermo-chemical processes (Das and Veziroglu, 2001; Dong *et al.,* 2009). The biological hydrogen production methods include photosynthetic and fermentative biohydrogen processes. Fermentative biohydrogen production is a more feasible process; for it can be carried out at ambient temperature and pressure without light and oxygen demand. Moreover, this process uses diverse microorganisms and waste materials for hydrogen production (Liu *et al.,* 2008; Pattra *et al.,* 2008).

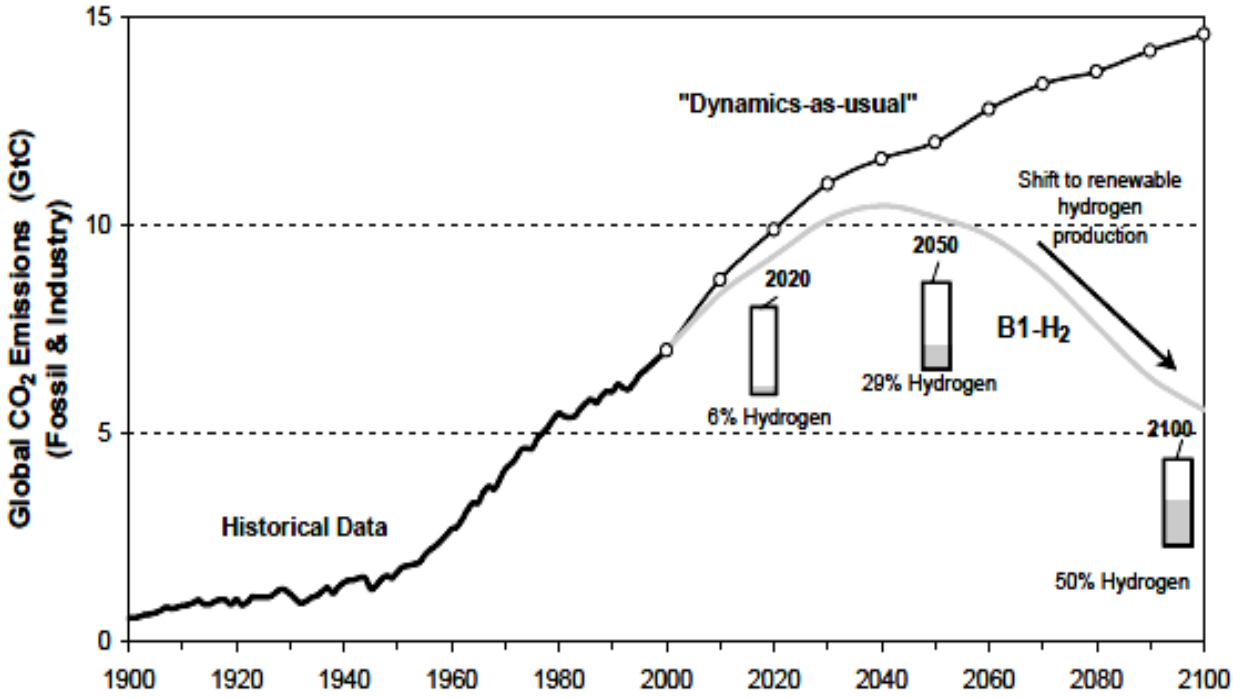

Figure 1.4: Global carbon dioxide emissions and hydrogen infrastructure development from 1900 to 2100 (Barreto *et al.*, 2003).

1.3. Problem statement

The energy crisis is one of the greatest challenges facing humanity, which includes escalating energy demands, dwindling fossil fuels and environmental degradation (Masilela, 2011). Microbial production of hydrogen has the potential to replace current technologies relying heavily on fossil fuels. However, its process development has been hindered by low conversion yields on substrates (Das and Veziroglu, 2001). Agro-municipal wastes are considered an economical source for biohydrogen production processes. This is attributed to the fact that these waste materials are abundant, easily hydrolysable, rich in carbohydrate content, and have a high hydrogen potential. Their disposal poses serious environmental hazards. Furthermore, optimizations of bioprocess parameters are essential for maximizing its production from these waste materials.

1.4. Aims

The aim of this work was to optimize the production of biohydrogen from dark fermentation of agro-municipal mixed wastes, examine the semi-pilot scale optimized process and further evaluate the bioelectricity generation efficiency from the process effluents.

To achieve this goal, the following specific objectives were carried out:

i. Modelling and optimization of hydrogen response on co-substrates of Organic Fraction of Solid Municipal Waste (OFSMW), Bean Husk (BH) and Corn Stalk (CS) using mixture design.

ii. Modelling and optimization of hydrogen response on operational setpoint parameters of pH, temperature, substrate concentration and Hydraulic Retention Time (HRT) using the optimized substrate above (i).

iii. Semi-pilot scale production of biohydrogen using substrate in (i) and optimized setpoint conditions in (ii).

iv. Bioelectricity generation from the process effluents of the semi-pilot above (iii) using a two-chambered Microbial Fuel Cell (MFC) process.

v. Review the impacts of miniaturized parallel bioreactors for biohydrogen process development.

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CHAPTER 2

Potential of using Organic Fraction of Solid Municipal Waste for fermentative biohydrogen production in South Africa

2.1. Abstract

Biohydrogen is believed to play a key role in the implementation of sustainable energy production, particularly when it is produced from renewable and low-energy processes. Organic Fraction of Solid Municipal Waste (OFSMW) is highly considered as a suitable substrate for fermentative biohydrogen production due to its nutritional contents. In addition, an estimated 7.88 million tonnes of organic waste was generated in 2011, and only 35% of these were recycled and the remaining poses an environmental challenge. In this review, the biohydrogen production potential of OFSMW is evaluated in light of recent data. The key physico-chemical parameters influencing biohydrogen production in dark fermentation of OFSMW are discussed. A comparative assessment of experimental biohydrogen production processes from OFSMW is examined. Finally, the economics of biohydrogen production from OFSMW is presented.

Keywords**:** Fermentative biohydrogen production, Organic Fraction of Solid Municipal Waste (OFSMW), Dark fermentation

2.2. Introduction

The use of fossil fuels as a primary energy source has led to serious energy crisis and environmental pollution on a global scale (Ni *et al.,* 2006). Therefore it is imperative to find alternative energy sources that are renewable and environmentally friendly. Hydrogen holds the potential as alternative fuel of the future due to its many social, economic and environmental benefits (Meher Kotay and Das, 2008). At present, 88% of commercial hydrogen is derived from thermochemical and electrochemical processes which involves the combustion of fossil fuels (Guo *et al.,* 2010). Moreover, these processes are highly energy consuming and are unsustainable. The global production of Organic Fraction of Solid Municipal Waste (OFSMW) is approximately 2 billion tonnes per year, and is predicted to increase to 3 billion tonnes by 2025 (Charles *et al.,* 2009). The production of OFSMW in South Africa is high and becoming a source of concern in municipal landfills because of its high organic matter content (DEA, 2012).

OFSMW has been recognized as a valuable resource that can be converted into useful products via microbial fermentation processes (Lesteur *et al.*, 2010; Yu and Huang, 2009). There are various methods available for the treatment of OFSMW but anaerobic digestion appears to be a promising approach (Lee *et al.*, 2009; Liu *et al.*, 2006a). It involves a series of metabolic pathways such as hydrolysis, acidogensis and methanogenesis (Themelis and Ulloa, 2007). Anaerobic digestion of OFSMW in landfills releases toxic gases such carbon dioxide that escape into the atmosphere and pollute the environment (Zhu *et al.*, 2008). But, under controlled conditions, the same process has the potential to provide clean and sustainable energy that does not require the supply of oxygen (Chanakya *et al.*, 2007; Guermoud *et al.*, 2009).

The use of OFSMW has been reported in literature (Dong *et al.*, 2009; Kim *et al.*, 2010; Lay *et al.*, 1999; Sekoai and Gueguim Kana, 2013; Shin *et al.*, 2004; Zhou *et al.*, 2012). It has a high carbohydrate content, wide availability and high hydrogen potential. Moreover, producing hydrogen from these waste materials greatly enhances the security of energy supply (De Vrije *et al.*, 2010) and is in accordance with sustainable development and waste minimization issues.

This study reviews the potential of using OFSMW for fermentative biohydrogen production in South Africa. The operational and process parameters affecting the anaerobic digestion of OFSMW are discussed. Finally, the economics of biohydrogen production from OFSMW is presented.

2.3. The South African generated OFSMW and disposal challenges

2.3.1. The generated OFSMW

The generated OFSMW are composed mainly of food waste, garden waste, paper, board, and other various types of waste materials (Albanna, 2013). The production and composition of OFSMW varies from place to place and from season to season. This is influenced by various factors such as geographic location, population's standard of living, energy source and weather conditions (Group, 2000). OFSMW is generated from various sources such as households, agricultural and industrial sectors.

The total waste distribution for South Africa in 2011 is shown in Table 2.1; an estimated 7.88 million tonnes of waste was produced by the municipal sector. The agricultural sector generated

2.95 million tonnes of waste and only 35% was recycled. The remaining was disposed on landfills. The amount of organic municipal waste generated by each province is presented in Table 2.2. It is evident from this data that South Africa is experiencing a significant growth in waste volumes. Consequently 42.2 million cubic metres of organic municipal waste was generated in 1997 and this value increased to 68.6 million in 2010. During this period, the production of organic waste rose up to 62.5% (DWAF, 2012). This is attributed to high level of industrialization and urbanization that is occurring in most cities across the country (DEA, 2012). Hence the production of OFSMW will have enormous pressure on municipalities across the country if it is not properly managed.

Table 2.1: Total waste distribution in South Africa (tonnes) (DEA, 2012).

Waste type	Generated	Recycled	Disposed	% Recycled
Municipal waste	7 878 564	-	7 878 564	0
Agricultural waste	2 954 461	1 034 061	1 920 400	35
Commercial and industrial waste	12 111 267	9 325 676	2 785 591	77
Brine	4 166 129	-	4 166 129	-
Fly ash and dust from miscellaneous filter source	31 420 488	1 885 229	29 535 259	6
Bottom ash	5 717 324	-	5 717 324	-
Slag	5 370 968	2 685 484	2 685 484	50
Mineral waste	369 000	-	369 000	-
Waste of electric and Electronic equipments	62 581	6 884	55 697	11
Sewage sludge	657 963	125 013	493 472	19
Miscellaneous	327 250	-	327 250	-
Construction and demolition waste	4 725 542	756 087	3 969 455	16
Paper	1 694 752	966 009	728 743	57
Plastic	1 278 713	230 168	1 048 545	18
Glass	937 869	300 118	637 751	32
Metals	3 121 203	2 496 962	624 241	80
Tyres	246 631	9 865	236 766	4
Other	36 171 127	-	36 171 127	0

DEA: Department of Environmental Affairs, -: data not available.

Table 2.2: South African distribution of municipal waste by provinces (DWAF, 2012).

	1997		2010		1997-2010	1997-2010
Province	**m^3**	**%**	**m^3**	**%**	**Total growth %**	**Annual average growth %**
Eastern Cape	2 281 000	5.4	3 105 989	4.5	36.2	2.6
Free State	1 674 000	4	3 877 380	5.6	131.6	7.3
Gauteng	17 899 000	42.4	26 085 304	38	45.7	3.2
KwaZulu-Natal	4 174 000	9.9	5 749 959	8.4	37.8	2.7
Limpopo	3 831 000	9.1	11 200 387	16.3	192.4	9.4
Mpumalanga	733 000	1.7	956 369	1.4	30.5	2.2
Northern Cape	1 470 000	3.5	2 374 864	3.5	61.6	4.1
North West	1 625 000	3.8	2 296 489	3.3	41.3	2.9
Western Cape	8 543 000	20.2	12 979 785	18.9	51.9	3.5
Total	42 230 000	100	68 626 526	100	62.5	4.1

DWAF: Department of Water Affairs and Forestry, m^3: cubic metres.

2.3.2. The challenges associated with the disposal of OFSMW

The disposal of OFSMW poses serious health risks on people living close to these sites. These landfill sites have been investigated as the possible cause of birth defects and respiratory illnesses such as asthma (Broomfield *et al.,* 2004). Incinerators have also been linked to these illnesses. Moreover, composting and material recycling facilities have been linked to odours and lung related diseases such as bronchitis (Broomfield *et al.,* 2004). Public health officials have raised concerns about the disposal of OFSMW, which has led to rats, flies, mosquitoes, and other disease vectors breed in open dumps, as well as in poorly constructed or poorly maintained housing facilities, in food storage facilities and in many other places where food waste is disposed (Tadesse, 2004).

In terms of environmental issues, decomposition reactions within the landfills produce large amounts of methane and carbon dioxide, which typically are vented to the atmosphere. The release of these gases through the landfill serves to carry out non-methane organic compounds that were originally present in the OFSMW or that were formed during decomposition (Eklund *et al.,* 1998). Viitez *et al.* (2000) reported that biotransformation of landfills occurs in a very slow process and may take several years to complete. They also reported that anaerobic fermentation processes on landfills may extend up to 20-40 years. And this poses serious detrimental effects on the environment. Moreover, the decomposition of OFSMW generates greenhouse gases such as methane and carbon dioxide which contributes to global warming. Methane is a major anthropogenic greenhouse gas, second to carbon dioxide in its impact on climate change and has a high global warming potential that is 25 times as large as the one of carbon dioxide (Kemfert and Schill, 2009). The disposal of OFSMW is expected to increase in developing nations than in less developed regions, this is due to rapid urbanization and industrialization that is occurring in these regions (Broomfield *et al.,* 2004).

2.4. Chemical composition of OFSMW

The composition of OFSMW varies from place to place but its chemical characteristics can be calculated by examining each component in the waste stream (Chen, 1995). OFSMW consists mainly of food waste which has high energy content and is highly biodegradable (Shin *et al.,* 2003). It contains 80-95% of volatile solids and 75-85% moisture, thus favouring microbial growth (Guo *et al.,* 2010). Tchobanoglous *et al.* (1993) reported the chemical properties of

various components of OFSMW as presented in Table 2.3, consisting of organic and inorganic materials. The organic materials such as food waste, paper, and cardboard comprises of large quantities. This favours microbial fermentation processes during anaerobic degradation of OFSMW.

Table 2.3: Chemical composition of OFSMW (Tchobanoglous *et al.*, 1993).

	Percentage by weight (dry basis)					
Component	**Carbon**	**Hydrogen**	**Oxygen**	**Nitrogen**	**Sulphur**	**Ash**
Organic						
Food waste	48	6.4	37.6	2.6	0.4	5
Paper	43.5	6	44	0.3	0.2	6
Cardboard	44	5.9	44.6	0.3	0.2	5
Plastics	60	7.2	22.8	-	-	10
Textiles	55	6.6	31.2	4.6	0.15	2.5
Rubber	78	10	-	2	-	10
Leather	60	8	11.6	10	0.4	10
Yard wastes	47.8	6	38	3.4	0.3	4.5
Wood	49.5	6	42.7	0.2	0.1	1.5
Inorganic						
Glass	0.5	0.1	0.4	<0.1	-	98.9
Metals	4.5	0.6	4.3	<0.1	-	90.5
Dirt, ash, etc.	26.3	3	2	0.5	0.2	68

-: data not available.

2.5. Suitability of OFSMW for biohydrogen production

OFSMW contains large proportions of organic and inorganic compounds. The latent energy present in OFSMW can be recovered via microbial fermentation processes to produce biohydrogen. The potential of using OFSMW for dark fermentation processes has been reported (Dong *et al.*, 2009; Elbeshbishy *et al.*, 2011; Kim *et al.*, 2004a; Lay *et al.*, 1999; Lin *et al.*, 2011b; Zhou *et al.*, 2012) with hydrogen yields of 134 ml/g VS, 97 ml/g VS, 122.9 ml/g VS, 180 ml/ g VS, 187 ml/g COD and 76 ml/g COD respectively. These studies were conducted at mesophilic temperatures (30-38 ^{o}C) and at different pH values (5-6).

In addition, foods processing wastewater from industries have a great potential for biohydrogen production due to their nutritional content. They contain high concentrations of carbohydrate-rich materials. For example, Molasses is a by-product of processed sugarcane or sugar beets and

often used as raw material by alcohol distilleries. Untreated molasses wastewater from alcoholic fermentation has a high organic content with chemical oxygen demand (COD) of 50–100 g/l (Jiménez *et al.*, 2004). Fructose wastewater is a by-product of many commercial products such as frozen foods, dairy products, and canned foods. It has a COD of 3000–6000 mg/l (Chao, 2004).

Lin *et al.* (2011b) studied the effect of temperature and pH on biohydrogen production from food processing wastewater of fructose and molasses using anaerobic mixed cultures and obtained a hydrogen yield of 166.8 ml H_2/g COD and 187 ml H_2/g COD respectively. Meanwhile Van Ginkel *et al.* (2005) investigated the production of hydrogen from confectioners, apple pomace, processed potato from industrial effluents and domestic wastewater. And reported a high hydrogen yield of 210 ml H_2/g COD from potato processing wastewater. Hence utilization of OFSMW presents a viable approach towards an economically feasible biohydrogen production processes.

Table 2.4: Hydrogen yields from selected agro-municipal wastes using microbial fermentation processes.

Inoculum	Type of substrate	H_2 yield	% H_2	Reference
Mixed cultures	OFSMW	180 ml/g TVS	66	Lay *et al.* (1999)
Thermoanaerobacterium	Food waste	0.9-1.8 mol/mol hexose	69	Shin *et al.* (2004)
Anaerobic sludge	Food waste + Sewage sludge	1.79 mol/mol hexose	-	Kim *et al.*(2011)
Mixed cultures	Food waste	122.9 ml/g COD	-	Kim *et al.* (2004a)
Anaerobic sludge	Rice	134 ml/g VS	57-70	Dong *et al.* (2009)
Anaerobic sludge	Potatoes	106 ml/g VS	41-55	Dong *et al.* (2009)
Anaerobic sludge	Lettuce	50 ml/g VS	37-67	Dong *et al.* (2009)
Mixed cultures	Rice waste	2.14 mol/mol hexose	53-61	Yu *et al.* (2002)
Mixed cultures	Biosolids	10-15 mg/ g COD	-	Wang *et al.* (2003b)
C. butyricum + E. aerogens	Sweet potato residue (5%)	7.0 mol/mol glucose	-	Yokoi *et al.* (2001)
C. butyricum + E. aerogens	Sweet potato residue (2%)	4.5 mol/mol glucose	-	Yokoi *et al.* (2002)
Mixed cultures	Fructose wastewater	166.8 ml/g COD	-	Lin *et al.* (2011b)
Mixed cultures	Molasses wastewater	187 ml/g COD	-	Lin *et al.* (2011b)
Anaerobic sludge	Food waste	205 ml/g VS	52-56	Chu *et al.* (2008)
Anaerobic sludge	OFSMW	52.5-71.3 N L/kg VS	-	Gomez *et al.* (2006)
Anaerobic sludge	Food waste	97 ml/g VS	-	Elbeshbishy *et al.* (2011)
Anaerobic digester	OFSMW	1-2.3 mol/mol hexose	43.9-51.4	Lee *et al.* (2010)
Anaerobic digester	Food waste	96-114 ml/g VS	-	Cappai *et al.* (2009)
Anaerobic sludge	OFSMW	76 ml/g COD	-	Zhou *et al.* (2012)

OFSMW: Organic Fraction of Solid Municipal Waste,-: data not available.

2.6. Operational and process parameters affecting the anaerobic digestion of OFSMW

2.6.1. Temperature

Temperature is one of the most significant parameters in biohydrogen fermentation processes. It affects the growth rate and metabolic pathways of biohydrogen-producing bacteria (Elsharnouby *et al.,* 2013). Thus, influences the activity of biohydrogen-producing enzymes such as hydrogenases during biohydrogen production, and affects parameters such as substrate utilization efficiency, hydrogen yields, volatile fatty acids production and microbial communities (Fang and Liu, 2002).

Biohydrogen fermentation processes are conducted at mesophilic (20-40 ^{o}C), thermophilic (40-65 ^{o}C) or hyperthermophilic conditions (>80 ^{o}C) (Sinha and Pandey, 2011). Published reports indicated that about 60% experiments were carried out with mesophilic cultures (Elsharnouby *et al.,* 2013). Thus, fermentation processes employing mesophilic conditions are desirable because they are less expensive. As a consequence, most studies of biohydrogen fermentation processes from food waste and OFSMW were conducted under mesophilic conditions (Boni *et al.,* 2013; Cappai *et al.,* 2009; Dong *et al.,* 2009; Kim *et al.,* 2010; Lee and Chung, 2010). Lin *et al.* (2011b) studied the effect of temperature (30-55 ^{o}C) on biohydrogen production from food processing wastewater, and obtained a two-fold increase in specific hydrogen production potential (SHPP) and maximum specific hydrogen production rate ($SHPR_m$) under thermophilic conditions (55 ^{o}C) than in mesophilic conditions. The optimum values for SHPP and $SHPR_m$ were 166.8 ml H_2/g COD and 26.7 ml H_2/g VSS h respectively. Kim *et al.* (2008) investigated the effect of mesophilic temperature (30-45 ^{o}C) on biohydrogen production using *Clostridium beijenckii* KCTC 1785. They observed that hydrogen production increased with increasing temperature. High amounts of volatile fatty acid components such as acetate and butyrate were produced at high temperatures.

The production of biohydrogen at thermophilic conditions has been reported (Azbar *et al.,* 2009; Ismail *et al.,* 2009; Munro *et al.,* 2009). Ismail *et al.* (2009) optimized biohydrogen production from food waste at thermophilic conditions (55.7 ^{o}C) using response surface methodology and obtained a yield of 120 ml H_2/g COD. Several studies have reported that thermophilic fermentations are favourable for fermentative biohydrogen production compared to mesophilic fermentations. This may be attributed to the fact that these processes provide better conditions

for inhibition of methanogenic bacteria (Kim *et al.,* 2004b; Lay *et al.,* 1999; Lin *et al.,* 2006a). Hydrogen yield and production rates of thermophilic bacteria, growing at temperature above 60 ^{o}C, often show higher values as compared to those of mesophilic bacteria growing at moderate temperatures (Schaefer *et al.,* 1999). However, there are specific constrains for hydrogen production by thermophiles and extreme thermophiles, one of them is associated with low bacterial cell densities, which result in rather moderate hydrogen productivities.

2.6.2. pH

pH is considered as the most pivotal parameters in fermentative biohydrogen production processes, due to its effect on the hydrogenase activity, metabolic activity, and substrate hydrolysis (De Gioannis *et al.,* 2013). The protons (H^{+}) ions are important for maintaining optimum levels of ATP and maintaining cell neutrality. Earlier studies reported that pH affects chemiosmosis in bacteria (Mitchell, 1961). It has been shown that bacterial membranes are sensitive to protons ions because they affect various activities within the cell such as the uptake of nutrients, pH gradient and polarity (Stouthamer, 1979). The inhibition of growth at a low pH may be due to insufficient energy to shift protons outwardly through the cell membranes to establish a proton motive gradient (Garland, 1977). In addition, enzymes are reported to be sensitive to protons; hence a proton load might inhibit the production of hydrogen.

Conflicting pH values ranging from 6-9 have been reported in literature for optimum biohydrogen production processes. This is due to substrate composition, inoculum used, and operating conditions. In most studies of biohydrogen fermentation, the initial pH is adjusted without further control. Comparative studies with regard to the effect of pH on fermentative biohydrogen production revealed that the optimum pH range for maximum hydrogen yield or specific hydrogen production rate was 5.2-6.0 using either pure or mixed cultures of bacteria (Oh *et al.,* 2004; Zhang *et al.,* 2008). Venkata Mohan *et al.* (2009) reported that the initial pH values of 5.5-7.5 may represent the optimum and acceptable range for biohydrogen production. Various studies have revealed that low pH values (below 4.5) inhibit the hydrogenase activity during dark fermentation process (Fang and Liu, 2002; Hawkes *et al.,* 2002; Khanal *et al.,* 2004).

The exponential growth phase of biohydrogen in clostridia occurs during the acidogenic process via acetate and butyrate fermentation pathways (Lay and Fan, 2003). This is often reported at pH

5.5-6.5 (Fang and Liu, 2002; Khanal *et al.,* 2004; Van Ginkel *et al.,* 2001). However during the process decline phase of hydrogen production, these a microbial transition from acidogenesis to solventogenesis process due to production of fermentative by-products such as volatile fatty acids (VFAs) and alcohols (Venkata Mohan *et al.,* 2008) which changes the buffering capacity of the medium, and is observed at pH below 4.5 (Khanal *et al.,* 2004).

Complete inhibition of biohydrogen production was reported at pH range of 4-5 in earlier studies by Bahl *et al.* (1986) and Roychowdhury *et al.* (1988). Hydrogen production at high pH values (above 6) has been reported (Abreu *et al.,* 2012; Bala Amutha and Murugesan, 2011; Chen *et al.,* 2012). A 14-fold biohydrogen production increase was observed by Bala Amutha and Murugesan (2011) when the pH was varied from 5 to 8. In other studies of biohydrogen fermentations, high pH values of 7 and 9 were reported to be ideal for its production (Abreu *et al.,* 2012; Lee *et al.,* 2002).

The optimum pH reported in literature for anaerobic digestion of food waste and OFSMW varies from 5.5-7.9. Maximum hydrogen yields of 134 ml H_2/g VS, 128 ml H_2/g COD, 43 ml H_2/g TVS, 56.74 ml/g TVS and 671 ml/g food waste have been obtained in dark fermentation processes operating within the pH ranges of 5.5 to 7.9 (Dong *et al.,* 2009; Kim *et al.,* 2008; Liu *et al.,* 2006a; Sekoai and Gueguim Kana, 2013; Zhong *et al.,* 2009). pH is usually controlled in pilot-scale studies of hydrogen fermentation processes using sensors and actuators (Chang *et al.,* 2011; Kim *et al.,* 2010; Lin *et al.,* 2011a). The control of pH during biohydrogen production is essential to prevent any metabolic shift and to suppress the biohydrogen-consuming bacteria while maintaining an enriched culture for biohydrogen-producing bacteria.

2.6.3. Hydraulic Retention Time (HRT)

HRT is considered an important control parameter affecting continuous production of biohydrogen (Zhang *et al.,* 2006a). The control of HRT in biohydrogen fermentation processes is necessary to inhibit the biohydrogen-consuming bacteria such as methanogens (Chen *et al.,* 2001). The optimum HRTs depends on the substrate used. Short HRTs are preferred in biohydrogen fermentation processes, they are known for suppressing the methanogenic bacteria since studies have shown that these bacterial species generally requires relatively longer times to grow as compared to acidogenic bacteria (Liu *et al.,* 2008). Short HRTs are viewed as cost-effective. Kim *et al.* (2004a) reported that short HRTs below 3 days enhance the production of

biohydrogen. It has been shown on various studies of biohydrogen fermentation processes that pH and HRT are joint parameters (Liu *et al.,* 2008; Shin and Youn, 2005; Zhang *et al.,* 2006b). Short HRTs result in low pH (Chang and Lin, 2004; Shin and Youn, 2005). Moreover, both these parameters have been viewed as effective for inhibition of biohydrogen-consuming bacteria at mesophilic and thermophilic conditions (Oh *et al.,* 2004). HRT controls the microbial growth and hence HRT must be greater than the maximum growth rate of organisms to prevent biomass washout (Hallenbeck and Ghosh, 2009).

Some studies have shown that HRTs ranging from 1-6 days are ideal for biohydrogen production (Liu *et al.,* 2008; Thanwised *et al.,* 2012; Zhang *et al.,* 2006a) and obtained yields of 21 ml/g VS, 883.19 H_2/L d and 1.6 mol H_2/g glucose respectively.

2.6.4. Organic Loading Rate (OLR)

Organic Loading rate (OLR) is a measure of biological conversion capacity of the anaerobic digestion process (Monnet, 2003). The OLR affects various fermentation conditions, such as the production of VFAs, COD removal efficiency, pH, as well as variations in the composition of the active biomass, with consequence modifications of the associated metabolic pathways (De Gioannis *et al.,* 2013). Shin and Youn (2005) observed that increasing OLR up to 8 g VS/L d while maintaining long HRT of 5 days enhanced the production of hydrogen. Hong and Haiyun (2010) maximized the production of biohydrogen when the OLR was increased from 4 to 8 g VSS/l d at long HRT of 8.92 days from food waste. A maximum hydrogen fraction and production rate of 57% and 5.4 L H_2/d were reported at OLR of 29 g COD /L d and 110 g TVS/ L d respectively by Tawfik and El-Qelish (2012) and Zahedi *et al.* (2012).

2.6.5. Bioreactor type and design

Different bioreactor configurations have been reported for fermentative biohydrogen production from waste. The size of these bioreactors varies from small-scale (100-500 ml) to semi-pilot scale (2-10 L) and are operated under batch, semi-continuous or continuous conditions (De Gioannis *et al.,* 2013; Show *et al.,* 2011). In an industrial context; continuous bioprocesses are recommended for assessment of various aspects such as monitoring the fermentation conditions, production and yield, and practical engineering design (Ismail *et al.,* 2009). The different bioreactor configurations used in biohydrogen production processes are discussed below.

2.6.5.1. Continuous stirred tank reactors

Continuous stirred tank reactors (CSTR) are known as backmix reactors and are commonly used in industrial fermentation processes. They consist of impellers and baffles which are used for agitation, and have an input and output flow (Baker and Gates, 1995). CSTRs are extensively used in biohydrogen production processes (Cappai *et al.,* 2009; Dong *et al.,* 2009; Gomez *et al.,* 2006; Kim *et al.,* 2011) due to effective homogenous mixing pattern. A good substrate-microbe contact and mass transfer is therefore accomplished in these reactors (Show *et al.,* 2011). They reach steady-state and demonstrate high efficiency and stable performance when the operational conditions are optimized (Won, 2013). But they cannot maintain high levels of biomass which is due to rapid mixing pattern. A schematic diagram of a CSTR is shown in Figure 2.1.

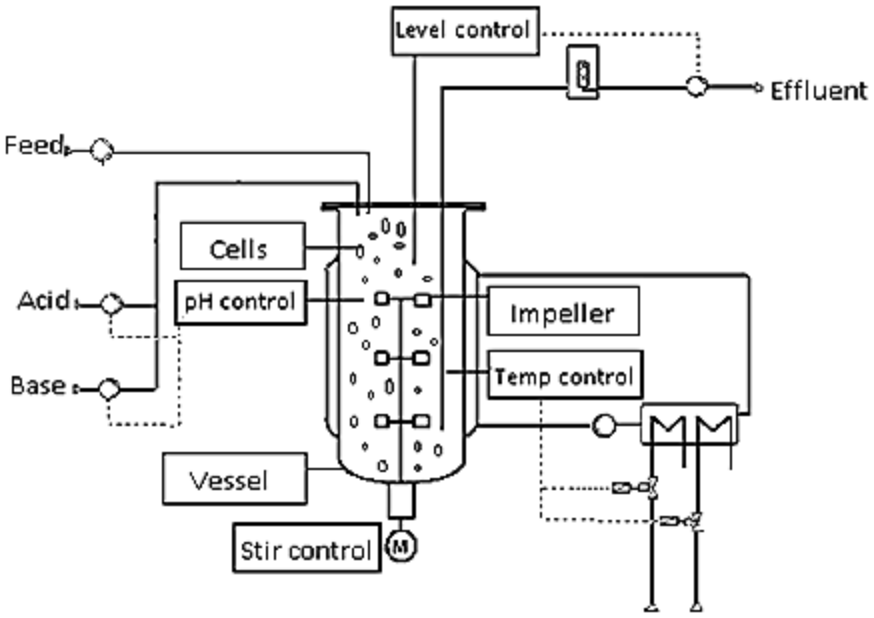

Figure 2.1: Schematic diagram of a CSTR (Fang and Liu, 2002).

2.6.5.2. Upflow anaerobic sludge blanket reactors

The development of upflow anaerobic sludge blanket reactors (UASBRs) was first proposed by Lettinga *et al.* (1980) in the early seventies for wastewater treatments. UASBRs are based on the development of granules formed by the natural self-immobilization of mixed microbial consortia. The feed enters at the bottom of the reactor via the inlet liquid distribution system and passes

upward through the dense anaerobic sludge bed. It was demonstrated that volumetric organic loading rates of more than 50 kg COD/m^3 d could be used because of high biomass concentration (Hulshoff Pol, 1989). The liquid velocity inside the reactor is usually in the range of 0.5–1.0 m/h. These reactors consist of a sludge bed, a sludge blanket and a three phase separator of weir, baffles and settler as shown in Figure 2.2. UASBRs are used in biohydrogen production processes because they can retain high biomass concentrations and often show high substrate conversion efficiency (Show *et al.*, 2006). The highest biohydrogen yield obtained from these reactors was 3.42 mol H_2/mol sucrose (Lo *et al.*, 2009).

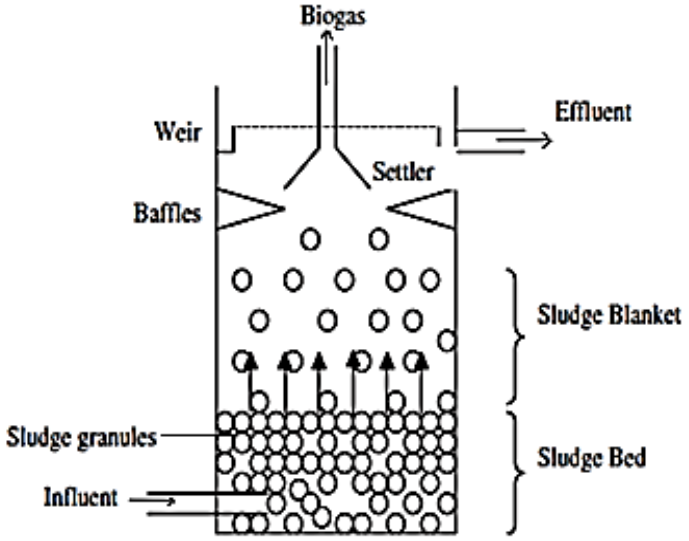

Figure 2.2: Schematic diagram of an UASBR (Saravanan and Sreekrishnan, 2006).

2.6.5.3. Anaerobic fluidized bed reactors

In anaerobic fluidized bed reactors (AFBRs), the feed is pumped through a bed of inert particles (with a size of 0.2–0.8 mm) at a sufficient velocity to cause fluidization (Nicolella *et al.*, 2000). Thus the media provides a large surface for attached biological growth and allows biomass concentrations to develop in the range of 10–40 kg/m^3 (Cooper and Sutton, 1983). AFBRs are favoured in biohydrogen production studies involving immobilized sewage sludge (Barros *et al.*, 2010; Chang *et al.*, 2002; Lin *et al.*, 2006b; Zhang *et al.*, 2007) because of high yields. These reactors are similar to packed bed reactors but the immobilized microbial consortia moves in a fluidized state (Won, 2013). Moreover, AFBRs have been shown to be more effective than other

high rate anaerobic reactors due to the following reasons, (i) they exhibits higher purification capacity, (ii) no clogging of the reactor, (iii) no problems of sludge washout and (iv) small volume and surface area requirements (Heijnen *et al.,* 1989). A typical flow diagram of an AFBR is shown in Figure 2.3.

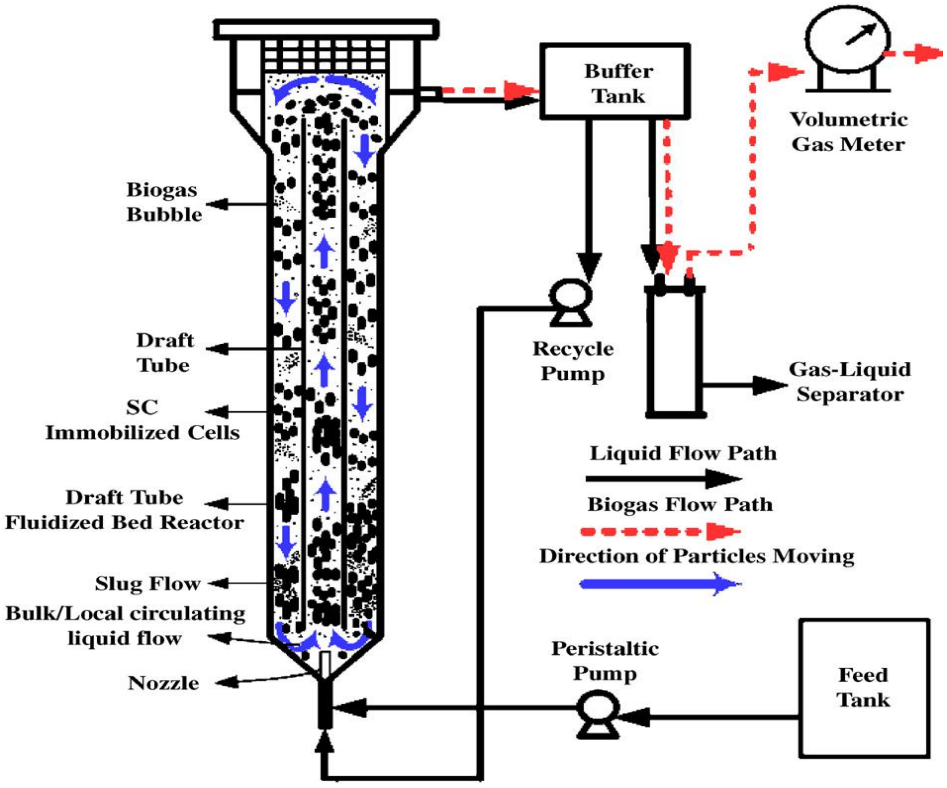

Figure 2.3: Schematic diagram of a draft tube FBR (Lin *et al.*, 2006b).

2.6.5.4. Anaerobic sequencing batch reactors

Biohydrogen fermentation studies have shown that anaerobic sequencing batch reactors (ASBRs) can maintain high biomass concentration compared to CSTRs (Buitrón and Carvajal, 2010; Kim *et al.,* 2008; Vijaya Bhaskar *et al.,* 2008). These types of reactors are characterized by the means of the physical retention of the microbial biomass and overcome the problem of washout, because microbial growth and the concentration of microbial biomass are considered independent of HRT. Vijaya Bhaskar *et al.* (2008) observed that biohydrogen production increased from 6.06 to 13.44 mol H_2/kg COD when the organic loading rate was increased from 6.3 to 7.9 kg COD/m^3 d. High cell concentrations can be achieved, fostering high volumetric production rates and high yields (Hallenbeck and Ghosh, 2009). Nonetheless, ASBRs may not

show higher productivity over CSTRs since they cannot reach steady-state and are semi-continuous (Won, 2013). A schematic representation of an ASBR is shown in Figure 2.4.

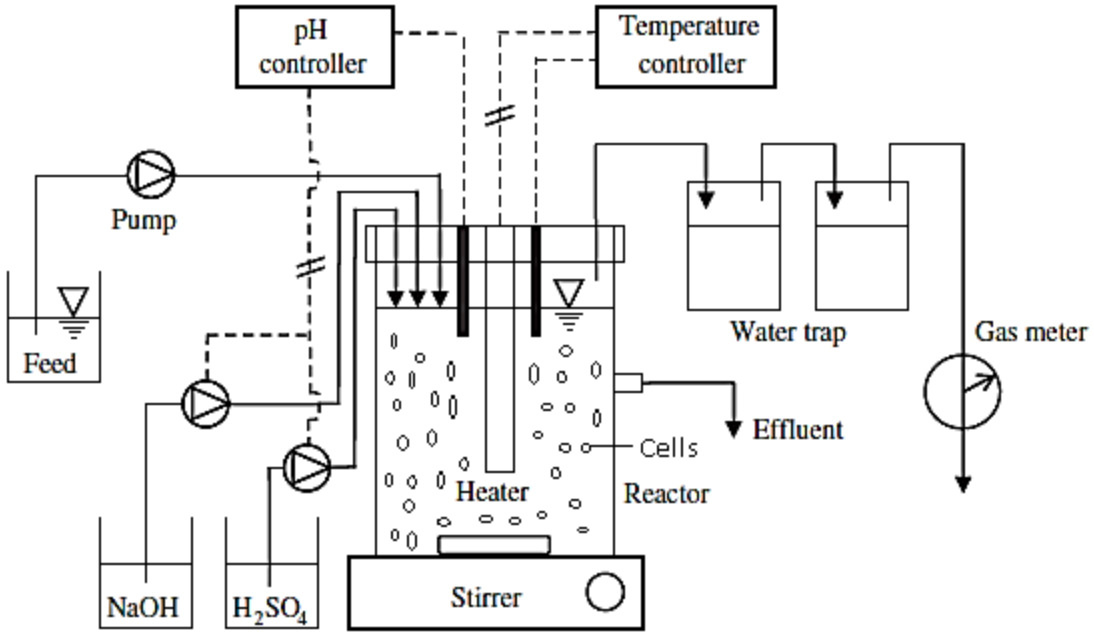

Figure 2.4: Schematic diagram of an ASBR (Searmsirimongkol *et al.*, 2011).

2.6.5.5. Membrane reactors

Amongst the biohydrogen producing reactors, membrane reactors (MRs) are recommended in biohydrogen fermentation processes because they possess the following advantages, (i) capital costs are reduced because of small reactor size (ii) the yields of biohydrogen are improved due to equilibrium shift effect and (iii) the costs of downstream processes are reduced because the separation is integrated (Gallucci *et al.*, 2013). Various types of membranes materials have been used in biohydrogen production studies; these include polymeric, porous, dense metal and proton conducting membranes (Gallucci *et al.*, 2013). Nevertheless studies have shown that dense metal (palladium alloys) and dense ceramic membranes are suitable for high purity hydrogen production, this is attributed to their hydrogen selectivity (Goldbach and Xu, 2011; Peters *et al.*, 2011; Zhang *et al.*, 2012). A membrane reactor was used in biohydrogen production process to control the biomass concentration (Oh *et al.*, 2004) at HRT of 3.3 hours. It was observed that an increase in sludge retention time of 2.2-5.8 g/l resulted to a concomitant increase in biohydrogen

production rate of 0.5 to 0.64 L H_2/h L. A schematic representation of a MR is shown in Figure 2.5.

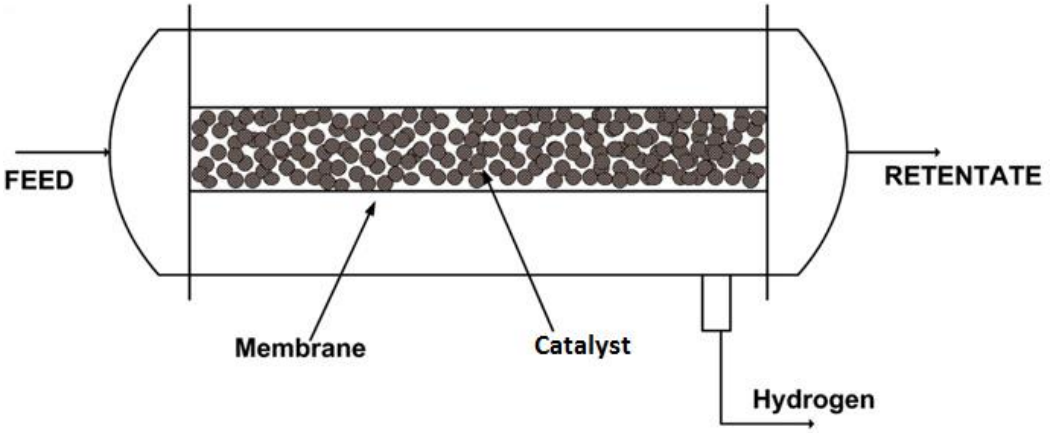

Figure 2.5: Schematic diagram of a MR (Gallucci *et al.*, 2013).

2.6.6. Type of inoculum

Biohydrogen production can be carried out using diverse microorganisms which are either pure or mixed cultures. Mixed cultures are isolated from diverse natural environments such as soil, wastewater sludge, compost and other various habitats. Many studies reported in literature for anaerobic digestion of food waste and OFSMW used mixed cultures (Fan *et al.*, 2004; Fang and Liu, 2002; Lay *et al.*, 1999; Morimoto *et al.*, 2004). Studies have also shown that utilization of mixed cultures improves the biohydrogen production efficiency (Abreu *et al.*, 2012; Lay *et al.*, 2012; Lin *et al.*, 2011a; Ozmihci and Kargi, 2010; Wang *et al.*, 2010). Hydrogen production by mixed culture fermentation is more suited for industrial applications, when compared to pure culture fermentation, due to the following reasons: (i) minimum sterility required, (ii) presence of high microbial diversity, which offers increased adaptation capacity, (iii) possibility of mixed substrates co-fermentation, (iv) higher capacity for continuous processing, and (v) utilization of diverse substrates (Kleerebezem and van Loosdrecht, 2007; Temudo *et al.*, 2007).

Microbial community analysis of various hydrogen producing activated systems showed that members of genus *Clostridium* are dominant and active hydrogen producers (Das and Veziroglu, 2001; Fang and Liu, 2002; Hung *et al.*, 2007; Wang and Wan, 2008). These bacterial species are gram positive, spore forming, and are rod-shaped obligate anaerobes. They are fastidious and can

utilize variety of substrates which is of great interest for industrial production of biohydrogen (Madigan *et al.,* 1997; Wang *et al.,* 2008). Their presence is reported to be more than 60% of total bacterial populations after pre-treatments (Pan *et al.,* 2008). This is possibly enhanced by the resistance of the spores (Fang *et al.,* 2006; Wang *et al.,* 2008). Several studies of biohydrogen fermentation processes have used *Clostridium* species, these includes *C. butyricum* (Yokoi *et al.,* 2001), *C. beijerinckii* KCTC 1785 (Kim *et al.,* 2008), *C. bifermentas* (Wang *et al.,* 2003a), and *C. tyrobutyricum* ATCC 25755 (Liu *et al.,* 2006b). Lin *et al.* (2007) studied the effect of four clostridial strains of *C. acetobutylicum* M121, *C. butyricum* ATCC19398, *C. tyrobutyricum* FYa102, and *C. beijerinckii* L9 respectively on biohydrogen production. They obtained a high yield of 2.81 mol/mol glucose.

Among the hydrogen-producing bacteria, members of the genus *Enterobacteriaceae* have also been reported for fermentative biohydrogen production (Khanna *et al.,* 2011; Kumar and Das, 2000; Ozmihci and Kargi, 2010; Tanisho *et al.,* 1987; Yokoi *et al.,* 1995). These bacterial species are facultative anaerobes, gram negative and rod-shaped organisms. They produce low hydrogen as compared to *Clostridium* species (Tenca *et al.,* 2011). Kumar and Das (2000) enhanced the production of hydrogen using *Enterobacter cloacae* IIT-BT 08 and achieved a maximum yield of 2.2 mol/mol glucose. Facultative anaerobic bacterium such as *Bacillus* species are also reported in literature (Liu and Wang, 2012; Manikkandan *et al.,* 2009; Meher Kotay and Das, 2008). Other hydrogen producing bacteria includes *Pseudomonas* sp., *Actinomyces* sp., *Streptococcus* sp., *Klepsiella* sp., *Eubacteria* and *Escherichia coli* (Hung *et al.,* 2007; Oh *et al.,* 2003). In pure cultures, metabolic pathways are easily detected due to the reduced diversity of the biomass. Moreover, studies employing pure cultures can reveal important information regarding conditions that promote high hydrogen yield and production rate (Elsharnouby *et al.,* 2013). However, using pure cultures has its own limitations such as strict sterilization procedures and the selectivity to substrates (Hawkes *et al.,* 2002).

The conversion of glucose to hydrogen by *Clostridium* species is associated with two metabolic pathways as shown in Figure 2.6. In the first pathway, pyruvate is converted to acetyl-CoA and CO_2 through pyruvate ferredoxin oxidoreductase (1) with the generation of reduced ferredoxin (Fd). Hydrogen is generated from the reduced Fd by the hydrogenase activity (3). The second pathway involves re-oxidizing part of the NADH produced during glycolysis by the NADH-

ferredoxin oxidoreductase (2) to produce reduced ferredoxin (Vardar-Schara *et al.*, 2008), which in turn is re-oxidized by the hydrogenase (3) to produce hydrogen. *Clostridium* species can stoichiometrically produce 2 and 4 mol H_2/mol glucose from butyrate and acetate-fermentation pathways respectively. However, the hydrogen yields are low due to formation of other fermentative by-products.

It was reported in some studies that butyrate pathway produces low yields because it has an inhibitory effects on hydrogen production (Chin *et al.,* 2003) and cell growth (Berrios-Rivera *et al.,* 2000). Moreover, it is recognized as the main competing pathway during hydrogen production because it utilizes more NADH than acetate pathway, this reduces the yield of hydrogen (Kumar *et al.,* 2001).

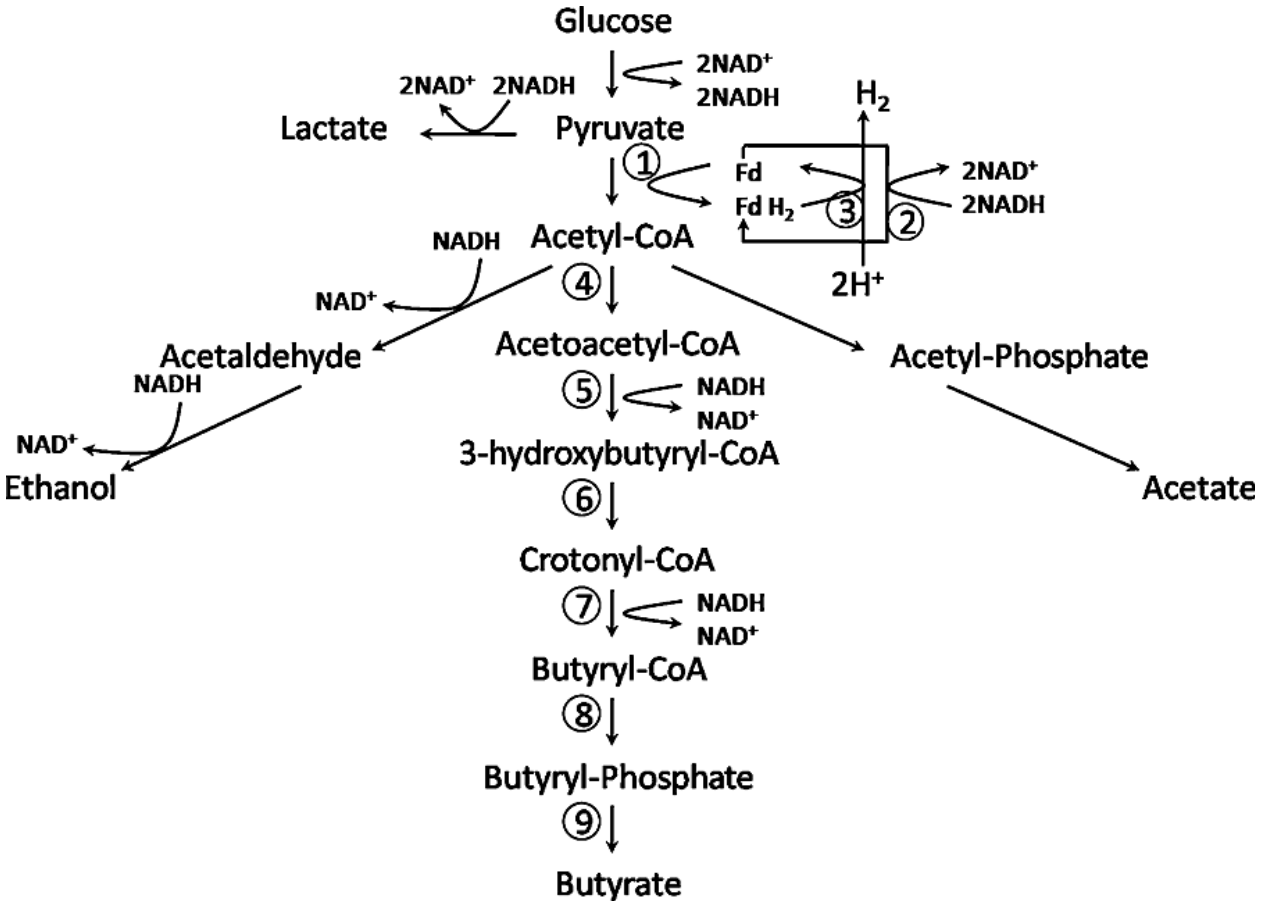

Figure 2.6: Metabolic pathways of *Clostridium* species, (1) pyruvate-ferredoxin oxidoreductase; (2) NADH-ferredoxin oxidoreductase; (3) hydrogenase; (4) acetyl-CoA acetyltransferase; (5) β-hydroxybutyryl-CoA dehydrogenase; (6) 3-hydroxybutyryl-CoA dehydratase; (7) butyryl-CoA dehydrogenase; (8) phosphotransbutyrylase; (9) butyrate kinase (Cai *et al.*, 2011).

Table 2.5: Operational process parameters setpoints reported in dark fermentation using food waste and OFSMW.

Substrate type	pH	Temperature (^{o}C)	HRT (h)	OLR	Reactor type	Reference
OFSMW	5	37	192	-	SR	Lay *et al.* (1999)
Food waste	5.6	35	120	-	SR	Shin *et al.* (2004)
Food waste	5.6	55	120	-	SR	Shin *et al.* (2004)
Food waste	6	35	30	-	SR	Kim *et al.* (2004b)
Rice waste	5.5	55	2-24	-	SR	Yu *et al.* (2002)
Rice waste	5.5	37	7	-	SR	Dong *et al.* (2009)
Potato starch	5.25	37	12	-	SR	Yokoi *et al.* (2002)
Food waste	5.5	37	60	-	SR	Zhou *et al.* (2012)
Food waste	6	34	0.8	-	SR	Gómez *et al.* (2009)
Food waste	5.5	30	21	7.4-11.7 g COD/L h	SR	Lee and Chung (2010)
OFSMW	5.7	38	24	-	UASBR	Alzate-Gaviria *et al.* (2007)
Food waste	5-6	37	48	45.7-45.9 g COD/L d	CSTR	Elbeshbishy *et al.* (2011)
Food waste	-	35	168	-	LBR	Han and Shin (2004)
OFSMW	5.56-5.95	55	504	-	SR	Valdez-Vazquez *et al.* (2005)
Household waste	5.2	37	1920	37.5 kg VS/m^3 d	SR	Liu *et al.* (2006a)
OFSMW	5.4-5.7	55	30-91	19.5-58.5 g COD/L d	SCR	Lee *et al.* (2010)
Food waste	5.5	37	64	-	SR	Zhou *et al.* (2012)

-: data not available, SR: Stirred Reactor, OLR: Organic Loading Rate, UASBR: Upflow Anaerobic Sludge Blanket Reactor, CSTR: Continuous Stirred Tank Reactor, LBR: Leaching-Bed Reactor, SCR: Semi-Continuous Reactor.

2.7. Economics of biohydrogen production from organic municipal wastes

Limited information on the economic analysis of dark fermentation process exists. Classen *et al.* (2000) examined the cost analysis for biohydrogen production using organic waste materials in thermo-bioreactor with a capacity of 95 000 L for dark fermentation and a photo-bioreactor with a capacity of 300 000 L equipped with sunlight collector. The size of the plant was set at production capacity of 39 kg H_2/h. Cost analysis showed an estimated overall cost of US \$3.65 kg^{-1} H_2. This estimation was based on assuming the cost of biomass as zero and zero hydrolysis costs; it excluded personnel costs and associated construction costs, all of which will influence the final price. Besides the final cost of generating hydrogen, the energy balance of this bioprocess was considered. The hydrogen production rate of 425 000 L H_2 h^{-1} was achieved from the process, this was equivalent to an energy production of 5.4 GJ h^{-1} (Classen *et al.*, 2000).

Benemann (2000) conducted a preliminary cost evaluation for biohydrogen production using microalgal system. The size of the reactor was 25694 kg H_2/day which was equivalent to 3600 GJ/day or 1.2 million GJ/year. The total capital costs for the reactor were estimated at US \$43 million, the annual operating costs was US \$12 million/year, and the total hydrogen production costs at US \$1.24 kg^{-1} H_2. In this analysis, the capital costs were approximately 90% of total costs at 25% annual capital charge. The costs of the algal reactor were estimated at US \$6 m^{-2}. The photo-bioreactors, with expected costs of US \$100 m^{-2}, were the major capital and operating cost factors, while the costs of gas handling were significant.

To fully realize the potential of fermentative biohydrogen production; two major barriers must be addressed. This includes the high cost of soluble sugars and the relatively low conversion efficiency. Glucose is the ideal substrate, yet it is too costly at present. Moreover, the challenge of using biomass lies in its crystallinity and heterogeneity, which prevents its direct utilization by most microbes. Physical and chemical pretreatment processes are therefore necessary to improve the yield. Even after pretreatment processes, the cellulose constituent still has to be further hydrolyzed via a suite of cellulase enzymes to produce the more fermentable glucose. Therefore utilization of waste materials may be a viable approach to overcome some of the economic constrains of biohydrogen process development.

2.8. Conclusion

Biohydrogen production processes from OFSMW demonstrate a feasible and attractive approach towards a sustainable energy development as these waste materials are abundant, renewable and inexpensive. Furthermore rapid industrialization, urbanization and economic activities in major cities across South Africa will increasingly generate more waste. This may have serious adverse effects on human health and the environment if these are not properly managed. Thus the production of biohydrogen from these waste materials will contribute to the generation of clean energy and mitigation of environmental pollution.

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CHAPTER 3

A two-stage modelling and optimization of biohydrogen production from a mixture of agro-municipal waste

3.1. Abstract

A two-stage modelling and optimization of biohydrogen production is reported. A mixture design was used to determine the optimum proportion of Bean Husk (BH), Corn Stalk (CS), and Organic Fraction of Solid Municipal Waste (OFSMW). The optimum operational setpoints for substrate concentration, pH, temperature and Hydraulic Retention Time (HRT) were further investigated using box-behnken design. The quadratic polynomial model from the mixture design had a coefficient of determination (R^2) of 0.9427 and the optimized mixtures were in the ratio of OFSMW: BH: CS = 30:0:0 and OFSMW: BH: CS =15:15:0 with yields of 56.47 ml H_2/g TVS and 41.16 ml H_2/g TVS respectively. Optimization on physico-chemical process parameters on the improved substrate gave the setpoints of 40.45 g/l, 7.9, 30.29 ^oC, 86.28 h for substrate concentration, pH, temperature and HRT respectively having a predicted H_2 yield of 57.73 ml H_2/g TVS. Model validation gave 58.62 ml H_2/g TVS, thus an improvement of 3.8% on the optimized mixture. Biohydrogen production can be significantly enhanced by a suitable mixture of agro-municipal waste and operation at optimal setpoints.

***Keywords*:** Bioprocess modelling and optimization, Fermentative biohydrogen production, Agricultural and municipal waste blends, Renewable energy, Mixture design

3.2. Introduction

The dependence on fossil fuels poses great challenges to both climate and environmental systems, thus prompting an urgent need for the development of non-polluting and renewable energy sources. Biohydrogen is an excellent alternative energy since its combustion produces only water. It has a high energy yield (122 kJ/g) which is 2.75 times greater than its equivalent of hydrocarbon fuels (Kapdan and Kargi, 2006; Das and Veziroglu, 2001). Its production via the fermentative route is more environmentally friendly, less energy intensive and hence being competitive to chemical hydrogen production methods (Lay *et al.*, 2012). Despite its many benefits, progress toward a biohydrogen economy has been hindered by a low yield on costly substrates.

Agricultural and organic municipal waste substrates are abundant, costless, renewable and can potentially be used as substrates for bioenergy production. An estimated annual yield of 118 $x10^9$ tons of dry biomass is generated worldwide (Rogalinski *et al.*, 2008), the energy equivalent of 60-70 billion tons of crude oil. South Africa generated 59 million tons of general wastes in 2011. The agricultural and municipal fractions were estimated at 2.95 and 7.88 million tons respectively, and only 35% of these, mainly of municipal types were recycled (DEA, 2012). The rest were burnt or disposed in landfills. Biohydrogen production using these substrates will not only alleviate environmental hazards but also save the energy demands needed to treat them. This work investigates the optimum proportion of Bean Husk (BH), Corn Stalk (CS) and Organic Fraction of Solid Municipal Waste (OFSMW) for biohydrogen production using mixture design. Furthermore the effects of input parameters of substrate concentration, pH, temperature and Hydraulic Retention Time (HRT) on hydrogen response using the mixed substrate are modelled and optimized.

3.3. Materials and methods

3.3.1. Determination of optimum substrate composition using mixture design

3.3.1.1. Mixture design and substrate pre-treatment

A mixture design was used to determine the optimum proportion of co-substrates of BH, CS and OFSMW for biohydrogen production. Fourteen different mixtures were generated with varied proportion of these substrates to a total concentration of 30 g/L (Table 3.1). The agricultural wastes of BH, CS were collected from the Ukulinga Research Farm, University of KwaZulu-Natal, South Africa. They were dried at room temperature, reduced in particles size to 2.00-2.80 mm, and kept for further use. OFSMW was simulated according to Gomez *et al.* (2006), and was made up of 10% apple, 10% orange, 35% cabbage, 35% potatoes, 8% bread, and 2% paper. The total volatile solids (TVS) content of experimental mixed crop residues was determined according to Equation (1).

$$\mathrm{TVS} = \frac{\text{Weight of dried waste} - \text{Weight of ash}}{\text{Weight of dried waste}} \times 100\% \qquad (1)$$

3.3.1.2. Inoculum development

Hydrogen-producing mixed consortia used in the study was obtained from the anaerobic sludge collected from the Darvill wastewater treatment plant, Pietermaritzburg, South Africa. Previous studies with this inoculum showed the presence of endospore forming clostridia (unpublished results). The sludge was heated at 100 °C for 30 minutes to deactivate the hydrogen consuming methanogenic bacteria, thus enabling the survival of hydrogen producing endospore forming bacteria.

3.3.1.3. Fermentation process

The fermentation processes were carried out in parallel bioreactors of 250 ml modified Erlenmeyer flasks. Reactors were fed with co-substrates at concentrations as stated in the mixture design to a total value of 30 g/L, supplemented with inorganic salts (all in g/L): NH_4Cl 0.5, KH_2PO_4 0.25, K_2HPO_4 0.25, $MgCl_2.6H_2O$ 0.3, $FeCl_3$ 0.025, $ZnCl_2$ 0.0115, $CuCl_2$ 0.0105, $CaCl_2$ 0.005 and $MnCl_2$ 0.015. They were inoculated with 10 ml of pre-treated sludge and made up to a working volume of 100 ml with distilled water. Anaerobiosis was created by flushing the reactors with nitrogen gas for 1 minute. The initial pH was adjusted to 6.5. Fermentations were carried out in duplicate in waterbath shaker with operational setpoints of 60 rpm, 35 ^{o}C and 72 hours for agitation, temperature and HRT respectively.

3.3.1.4. Analytical procedure

The evolving biogas volume was measured using the water displacement method (Veena *et al.,* 2012). This method is reliable and offers the possibility of being interfaced with a computer module. The hydrogen fraction of mixed biogas was determined using the hydrogen sensor BCP-H_2 (Bluesens, Germany) with a range of 0-100% and a measuring principle based on thermal conductivity detector. The cumulative volume of biohydrogen produced was computed regularly according to Equation (2).

$$V_{H,i} = V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V_H (C_{H,i} - C_{H,i-1}) \quad (2)$$

$V_{H,i}$ and $V_{H,i-1}$ are cumulative hydrogen gas volume at the current (i) and previous (i-1) time intervals, $V_{G,i}$ and $V_{G,i-1}$ the total biogas volumes in the current and previous time intervals, $C_{H,i}$ and $C_{H,i-1}$ the fraction of hydrogen gas in the headspace of the reactor in the current and previous time intervals, and V_H the total volume of headspace in the reactor (Chong *et al.,* 2009).

3.3.1.5. Modelling and optimization of mixtures

The experimental data were used in multiple regression analysis to develop a quadratic model that relates hydrogen production to the proportions of BH, CS and OFSMW in the mixture according to Equation (3).

$$Y=\alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_3 + \alpha_{11} x_1^2 + \alpha_{22} x_2^2 + \alpha_{33} x_3^2 + \alpha_{12} x_1 x_2 + \alpha_{13} x_1 x_3 + \alpha_{23} x_2 x_3 \quad (3)$$

Where Y is the hydrogen response, α_0 is the intercept, $\alpha_1 x_1$ to $\alpha_3 x_3$ represents linear blending portion, $\alpha_{11} x_1^2$ to $\alpha_{33} x_3^2$ are quadratic coefficients and $\alpha_{12} x_1 x_2$ to $\alpha_{23} x_2 x_3$ are the interaction coefficients. The significance of the model was assessed by the Analysis of Variance (ANOVA) using Design Expert software, (Stat Ease, Inc.). The optimum proportion of the co-substrates in the mixture was obtained by solving the quadratic equation. The optimum substrate concentration and other physico-chemical process variables were subsequently investigated using the box-behnken design.

3.3.2. Determination of optimum parameter setpoints using box-behnken design

3.3.2.1. Experimental setup

Box-Behnken design was used to model the relationship between the physico-chemical variables of substrate concentration, pH, temperature and HRT on hydrogen response, and to determine the optimum operational setpoints. Twenty nine fermentation batches with varied combination of input parameters were generated (Table 3.4) for experimentation. Parallel bioreactors made up of modified Erlenmeyer flasks were fed with the previously optimized medium, inoculated with 10 ml of pre-treated sludge and made up to 100 ml with distilled water. Fermentation processes were carried out as described in the previous stage, but with the physico-chemical parameters varied according to the box-behnken design.

3.3.2.2. Modelling and optimization of physico-chemical variables

The experimental data obtained from this stage were used in multiple regression analysis to develop a quadratic model that relates hydrogen production to the considered physico-chemical parameters. This model was subjected to the ANOVA. The optimum operational conditions for H_2 production were obtained by solving the Equation 3.

3.4. Results and discussion

3.4.1. Process model on co-substrate inputs

Experimental data from the mixture design (Table 3.1) were used to fit a quadratic model relating the OFSMW, BH and CS to hydrogen production. Analysis of variance of the model (Table 3.2) gave a coefficient of determination of 0.94, thus 94% of the variation in observed data can be explained by the model. The significance of the model was confirmed by the F and P values of 26.32 and 0.0001 respectively. The model can be mathematically expressed according to Equation (4).

Table 3.1: Biohydrogen production from mixture design.

Batch	A: OFSMW (g/l)	B: Bean Husk (g/l)	C: Corn Stalk (g/l)	H_2 yield (ml/g TVS)
1	30	0	0	56.47
2	5	5	20	11.57
3	0	30	0	17.67
4	0	15	15	12.73
5	20	5	5	40.54
6	15	15	0	33.4
7	15	15	0	23.75
8	30	0	0	54.22
9	0	0	30	3.9
10	10	10	10	16.37
11	15	0	15	24.05
12	0	0	30	3.68
13	5	20	5	14.56
14	0	30	0	31.04

Table 3.2: Analysis of variance generated from mixture design.

Source	Sum of Squares	df	Mean of Squares	F-Value	P-value	R-square
Model	2132.92	54	26.58	26.32	0.0001	0.9427

df: degrees of freedom, **F-value:** Fisher-Snedecor distribution value, **P-value:** Probability value, **R-square:** Coefficient of determination.

$$Y = +44.32A + 18.62B + 3.07C - 36.57AB - 15.34AC - 10.20BC \quad (4)$$

Where Y represents H_2 production in ml H_2/g TVS. The coefficient of estimates are shown in Table 3.3, where A, B and C are the linear coefficients of OFSMW, BH and CS respectively and AB, AC and BC are the interactive coefficient of OFSMW and BH, OFSMW and CS, and BH and CS respectively.

Table 3.3: Coefficients of estimates of the mixture model and their confidence intervals.

Component	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
A	44.32	1.00	2.79	37.88	50.77	1.62
B	18.62	1.00	2.79	12.18	25.07	1.62
C	3.07	1.00	2.79	-3.37	9.50	1.50
AB	36.57	13.22	1.00	-67.06	-6.09	1.76
AC	15.34	15.84	1.00	-51.88	21.19	1.55
BC	10.20	15.84	1.00	-46.74	26.34	1.55

df: degrees of freedom, **95% CI Low:** 95% Confidence Intervals (Low limit), **95% CI High:** 95% Confidence Intervals (High limit), **VIF:** Variance Inflation Factor.

3.4.2. Interaction of co-substrates on biohydrogen output and optimization

The hydrogen production from various mixtures, under similar fermentation conditions ranged from 3.68 to 56.47 ml H_2/g TVS (Table 3.1). This emphasizes the sensitivity of biohydrogen fermentation on substrate composition, as observed earlier by Zhang *et al.* (2007). Hydrogen yields of 56.47, 31.04 and 3.9 ml H_2/g TVS were obtained when OFSMW, BH and CS were used as sole substrate respectively, and a consistent high hydrogen production was observed in various mixtures containing the OFSMW (batch 5, 6, 7 and 11). A plausible contribution to a high hydrogen production on OFSMW might be its relative higher nutritional composition. A similar high hydrogen production pattern on OFSMW was observed by Dong *et al.* (2009), and was attributed to its rich contents of carbohydrates, lipids and proteins required for hydrogen

production. A 14 times decrease in H_2 production was obtained when comparing CS to OFSMW as sole substrate for fermentative H_2 production. This relative low yield on CS may be linked to the complexity of the polymer structure requiring an acidic or thermal pretreatment, which at industrial scale might substantially impact on process economics. With a HCl pretreatment of CS at 90 ^{o}C for 2 hours, Wung *et al.* (2010) achieved hydrogen yield of 126.22 ml/g CS. These observations might suggest that a pretreated CS releases higher amount of soluble sugars into the medium than OFSMW, but however the pattern and the cost/benefit analysis will need to be investigated.

The interactive effect of the mixture on hydrogen response is illustrated on triangular response surface graph and the contour map plot (Figures 3.1a and b). It is observed that hydrogen production was maximum in a mixture having highest concentration of OFSMW and progressively decreased along the axes OFSMW-BH and OFSMW-CS. A very low hydrogen response was obtained when BH and CS alone were used in the mixture, even at any proportion. The optimum proportion of OFSMW, BH and CS for hydrogen production was determined by solving the quadratic model equation using the numerical method of Myers and Montgomery (1995). Two solutions were selected: A mixture of 15 g/l OFSMW, 15 g/l BH and 0 g/l CS predicting a cumulative H_2 production of 41.16 ml H_2/g TVS, and a mixture of 30 g/l OFSMW, 0 g/l BH and 0 g/l CS with a cumulative H_2 production of 56.47 ml H_2/g TVS. It is expected that a viable production of biohydrogen at a large scale will depend on the distribution and availability of waste substrate types; hence under certain conditions a mixture of OFSMW and BH may be used instead of OFSMW as unique substrate. However in this study further optimization was based on OFSMW as sole substrate as derived from the optimized mixture design.

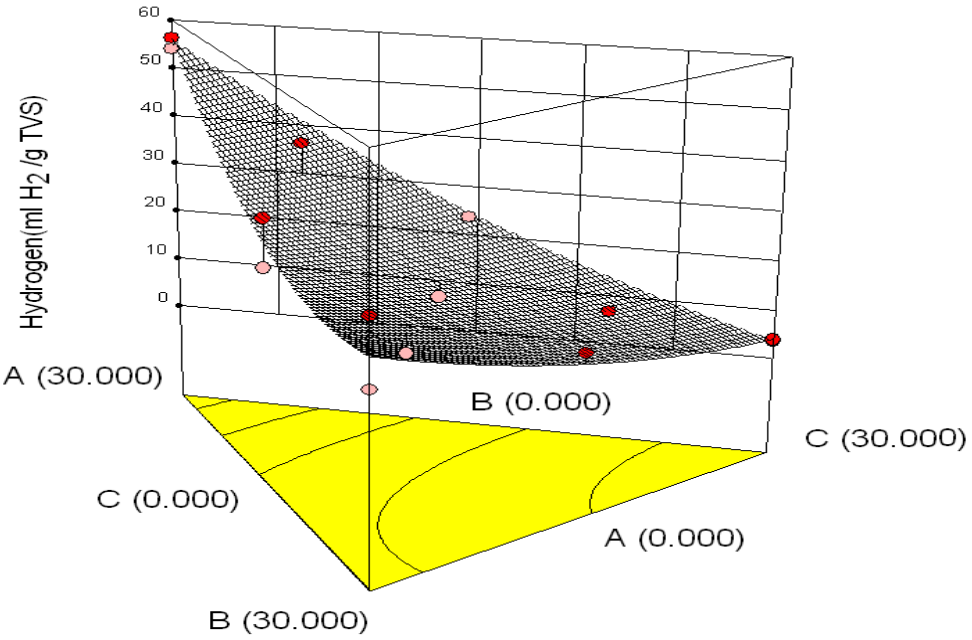

Figure 3.1a: Hydrogen response surface graph from mixture.

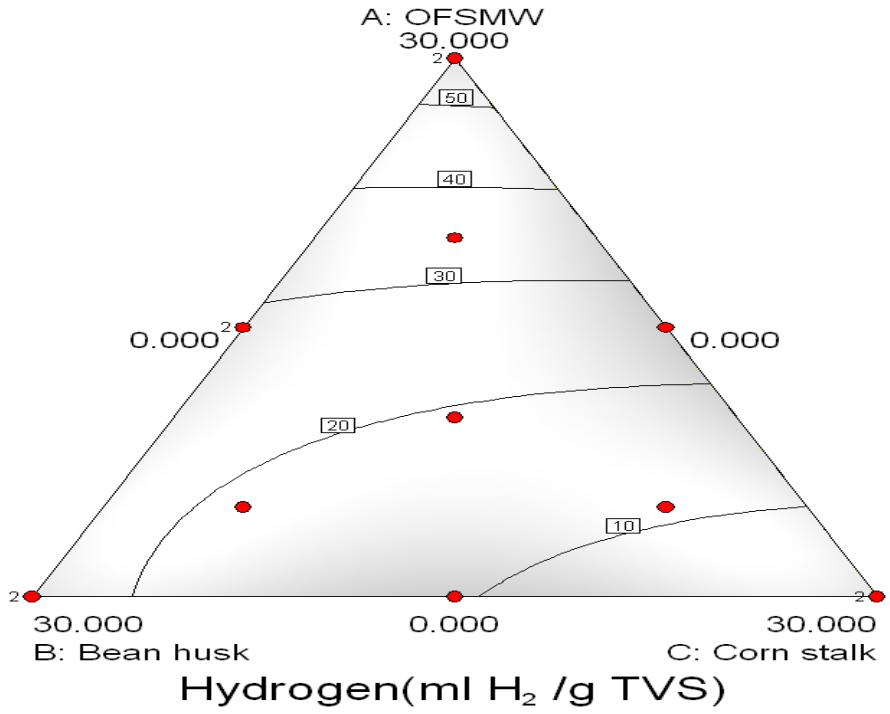

Figure 3.1b: Hydrogen response contour plot from mixture.

3.4.3. Process model based on physico-chemical input parameters

Experimental data obtained from box-behnken design (Table 3.4) were used to develop a second order polynomial Equation 5, whose coefficients were determined by multiple regression analysis. The suitability of the model was assessed using the ANOVA (Table 3.5). The high model F value (3.77) and low P value (0.0092) imply that the model is significant. A coefficient of determination R^2 of 0.7903 was obtained, thus 79.03% of the variability observed in the data can be accounted for by the model. The model's coefficient of estimates are shown in Table 3.6, where A, B, C, D are the linear coefficients for substrate concentration, HRT, pH and temperature. The magnitude of the coefficient has a direct contribution to the model output. Hence, C, BC, AC and B with coefficient values of 11.72, 11.28, 9.0 and 5.80 have a greater impact on hydrogen response compared to the remaining linear and interactive input effects. This model was expressed mathematically according to Equation (5).

Table 3.4: Biohydrogen production from box-behnken design.

Batch	Substrate conc. (g/l)	HRT	pH	Temperature (^{o}C)	H_2 yield (ml H_2/g TVS)
1	50	53	3	34.5	14.95
2	32.5	53	8	39	7.09
3	50	53	5.5	30	5.95
4	32.5	53	5.5	34.5	41.44
5	32.5	10	3	34.5	0.077
6	32.5	53	8	30	57.65
7	15	96	5.5	34.5	10.11
8	32.5	53	5.5	34.5	15.64
9	32.5	53	5.5	34.5	10.2
10	50	10	5.5	34.5	0.431
11	32.5	53	5.5	34.5	30.55
12	50	53	5.5	39	2.89
13	32.5	53	3	30	0.526
14	32.5	53	3	39	0.676
15	15	53	3	34.5	14.30
16	15	53	8	34.5	11.41
17	32.5	10	5.5	39	0.545
18	32.5	96	5.5	39	0.264
19	32.5	96	5.5	30	14.66
20	15	53	5.5	39	0.222
21	32.5	96	3	34.5	0.158
22	50	53	8	34.5	48.08
23	15	10	5.5	34.5	0.258
24	50	96	5.5	34.5	1.13
25	15	53	5.5	30	1.2
26	32.5	10	5.5	30	0.583
27	32.5	53	5.5	34.5	32.38
28	32.5	96	8	34.5	46.18
29	32.5	10	8	34.5	0.973

Table 3.5: ANOVA of the box-behnken derived model.

Source	Sum of Squares	df	Mean of Squares	F-Value	P-value	R-square
Model	6366.59	14	454.76	3.77	0.0092	0.7903

df: degrees of freedom, **F-value:** Fisher-Snedecor distribution value, **P-value:** Probability value, **R-square:** Coefficient of determination.

$$Y = +26.04 + 2.99A + 5.80B + 11.72C - 5.74D - 2.29AB + 9.00AC - 0.52AD + 11.28\,BC - 3.59BD - 12.68CD - 9.17A^2 - 13.61B^2 + 2.23C^2 - 11.50D^2 \quad (5)$$

Where Y is the hydrogen yield in ml H_2/g TVS; A, B, C and D are linear coefficients, AB to CD are the interactive coefficients of parameters on hydrogen production and A^2 to D^2 are the quadratic coefficients.

Table 3.6: Coefficients of estimates for the box-behnken model and their confidence intervals.

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	26.04	1	4.91	15.50	36.58	
A	2.99	1	3.17	-3.81	9.80	1.00
B	5.80	1	3.17	-1.00	12.60	1.00
C	11.72	1	3.17	4.92	18.52	1.00
D	-5.74	1	3.17	-12.54	1.06	1.00
AB	-2.29	5.49	1	-14.07	9.49	1.00
AC	9.00	5.49	1	-2.78	20.78	1.00
AD	-0.52	5.49	1	-12.30	11.26	1.00
BC	11.28	5.49	1	-0.50	23.06	1.00
BD	-3.59	5.49	1	-15.37	8.19	1.00
CD	-12.68	5.49	1	-24.46	-0.90	1.00
A^2	-9.17	4.31	1	-18.42	0.083	1.08
B^2	-13.61	4.31	1	-22.86	-4.36	1.08
C^2	2.23	4.31	1	-7.02	11.48	1.08
D^2	-11.50	4.31	1	-20.75	-2.25	1.08

df: degrees of freedom, **95% CI Low:** 95% Confidence Intervals (Low limit), **95% CI High:** 95% Confidence Intervals (High limit), **VIF:** Variance Inflation Factor.

3.4.4. Interaction of physico-chemical parameters on hydrogen production

Biohydrogen yield under different physico-chemical parameters varied from 0.077 to 57.65 ml H_2/g TVS (Table 3.4). Analysis of linear effect of parameters on hydrogen yield pattern indicated that at low setpoint values of HRT, pH, temperature and substrate concentration, low yields of hydrogen were obtained (batch 5, 17 and 23). The interaction of various physico-chemical parameters on hydrogen response taken pairwise with other parameter setpoints maintained at their median values are shown on three dimensional response surface graphs (Figures 3.2-3.7).

In Figure 3.2, the interactive effects of HRT and substrate concentration on hydrogen response has a concave shape indicating that the optimum setpoints were within the search range, and a peak production above 20 ml H_2/g TVS was observed within the ranges of 48-87 h and 20-42 g/l

of HRT and substrate concentration respectively. Fan *et al.* (2006) reported a remarkable increase in H_2 yield with the increase in substrate concentration in the range of 5-20 g/l. But it is believed that at a very high substrate concentration, the accumulation of volatile fatty acids increases, in addition hydrogen high pressure inhibits the hydrogenase activity (Fan *et al.*, 2006).

The synergistic effect of pH and substrate concentration (Figure 3.3) showed that at pH value between 7-8, an increase of OFSMW concentration from 20 to 42 g/l resulted in a more hydrogen production. Conflicting optimum pH setpoint values ranging from 6-9 have been reported for fermentative biohydrogen production. This might be attributed to the experimental setup, as very often only the initial pH value is reported without further control feedback or buffer system to stabilise the setpoint, despite the fact that fermentation processes are known to exhibit a highly nonlinear pH behaviour as function of inoculum source and substrate type. Moreso, even when pH control additives are intermittently used in shake flasks, it is not known how fast these liquid additions are mixed with the broth due to the poor mass transfer in these systems, and it has been demonstrated that microorganisms can swiftly change their metabolic fluxes within a time scale of less than a second (Fang and Liu, 2002; Ginkel *et al.*, 2001; Schaefer *et al.*, 1999).

Considering the process temperature and substrate concentration, it was observed that at temperatures above 30 ^{o}C, a further increase of substrate feed from 15 g/l resulted in an increase in biohydrogen production (Figure 3.4). Temperature affects the maximum specific growth, substrate utilization rate and the metabolic pathway of microorganisms, resulting in a shift of by-product compositions (Lay, 2000; Lin and Fang, 2007; Lin *et al.*, 2006). Several studies have reported that thermophilic fermentations are favourable for H_2 production compared to mesophilic fermentations. This may be attributed to the fact that these conditions lower the growth rate of hydrogen consuming bacteria (Kim *et al.*, 2005; Lay *et al.*, 1999; Schonheit and Schafer, 1995). Hydrogen yield and production rates of thermophilic bacteria, growing at temperature above 60 ^{o}C, often show higher values as compared to those of mesophilic bacteria growing at moderate temperatures (Chen and Lin, 2003; van Groenestijin *et al.*, 2002). Nevertheless, there are specific constrains for H_2 production by thermophiles and extreme thermophiles, one of them is associated with low bacterial cell densities, which result in rather moderate H_2 productivities. As illustrated in Figure 3.5, at pH value about 7, an increase in HRT within the window of 55-87 h resulted in more hydrogen yield. At pH below 4, very low

hydrogen is produced, even at any HRT value. A peak hydrogen production was obtained within a window of 60-87 h, 30-35 ºC for HRT and temperature respectively (Figure 3.6). With regards to temperature and pH, it can be observed in Figure 3.7 that at temperature values slightly above 35°C, a gradual increase in process pH from 6-8 leads to a growth in hydrogen. At temperature beyond 37°C, a gradual increase in pH does not improve the production of biohydrogen.

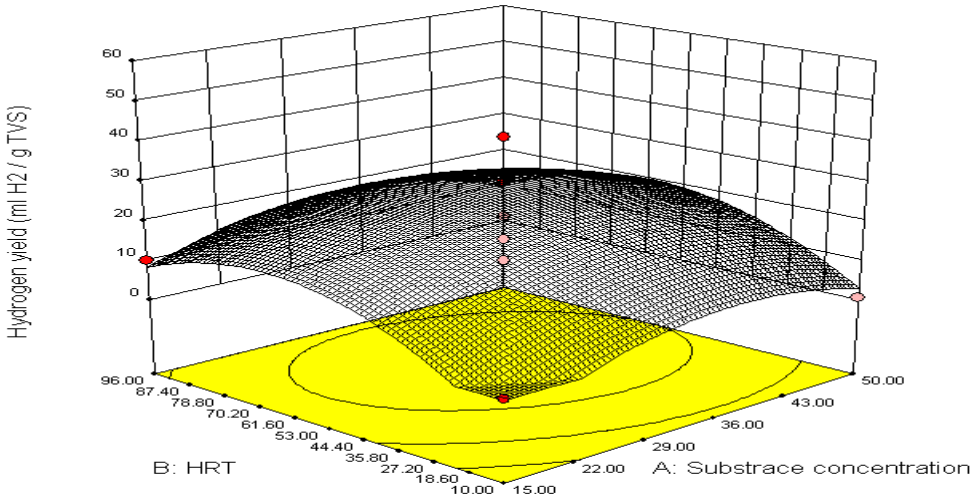

Figure 3.2: Hydrogen response surface graph exhibiting the interactive effects between HRT (h) and substrate concentration (g/l). Other variables were held at their median values.

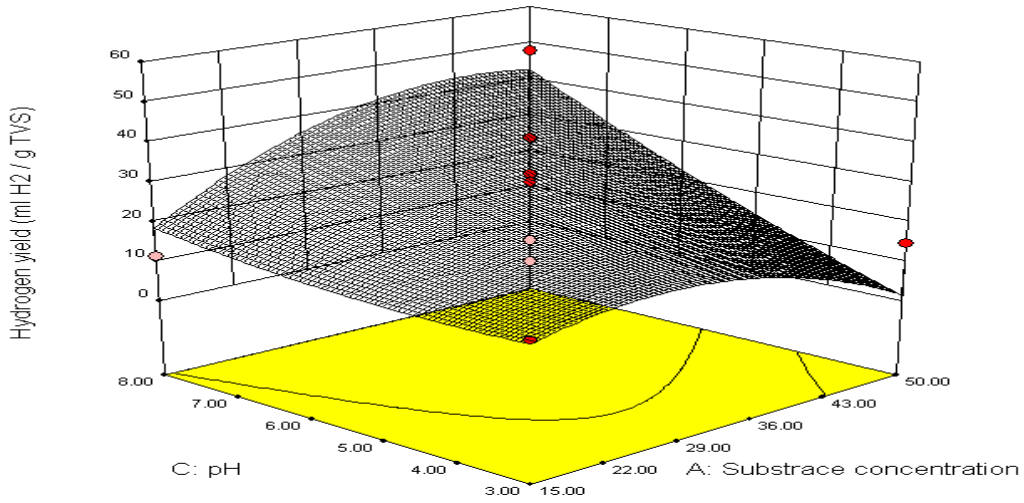

Figure 3.3: Hydrogen response surface graph exhibiting the interactive effects between pH and substrate concentration (g/l). Other variables were held at their median values.

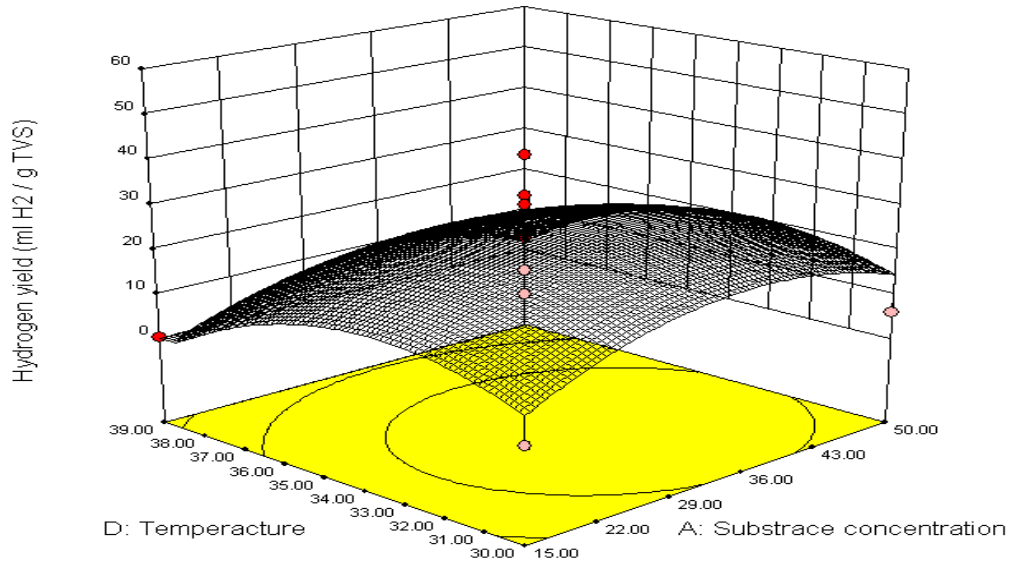

Figure 3.4: Hydrogen response surface graph exhibiting the interactive effects between temperature (^{o}C) and substrate concentration (g/l). Other variables were held at their median values.

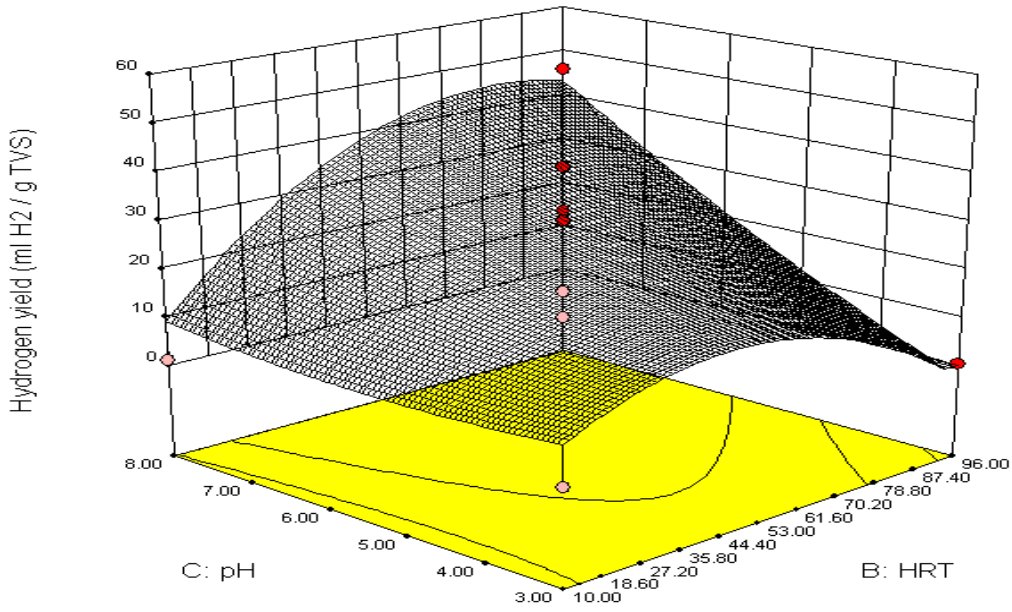

Figure 3.5: Hydrogen response surface graph exhibiting the interactive effects between pH and HRT (h). Other variables were held at their median values.

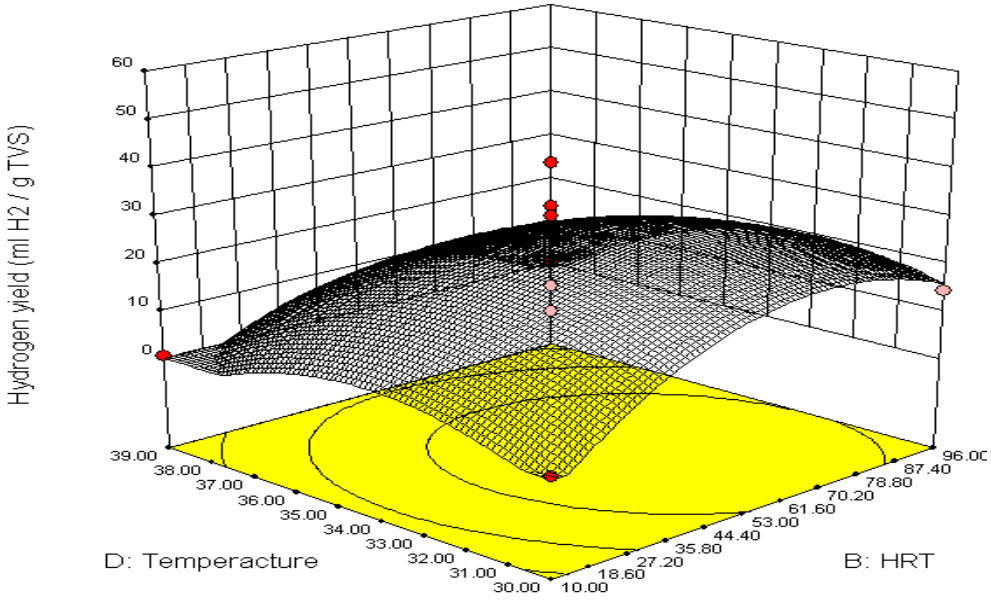

Figure 3.6: Hydrogen response surface graph exhibiting the interactive effects between temperature (^{o}C) and HRT (h). Other variables were held at their median values.

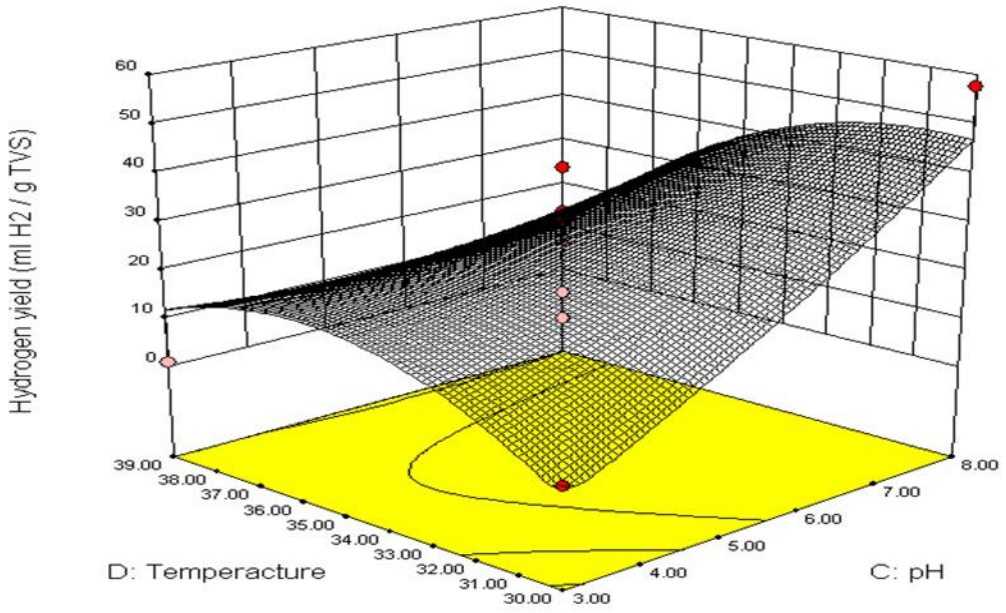

Figure 3.7: Hydrogen response surface graph exhibiting the interactive effects between temperature (^{o}C) and pH. Other variables were held at their median values.

3.4.5. Optimization of biohydrogen production using box-behnken design

The optimum operational setpoints of physico-chemical parameters were 40.45 g/l, 86.28 h, pH 7.9 and 30.29 ^{o}C for substrate concentration, HRT, pH and temperature respectively predicting a yield of 57.73 ml H_2/g TVS on hydrogen. The experimental validation gave 58.62 ml H_2/g TVS, thus 3.81% improvement on the optimized substrate.

3.5. Conclusion

A two-stage modelling and optimization of biohydrogen production on agro-municipal wastes of BH, CS, OFSMW and the associated operational parameters was carried out. The study revealed that without a prior treatment of substrates, a high yield of biohydrogen could be achieved using optimized mixtures in the ratio of OFSMW: BH: CS = 30:0:0 or OFSMW: BH: CS =15:15:0 with process operation at optimum setpoints conditions. An initial optimization of wastes substrate mixture, followed by appropriate combination of optimum operational variables enhances fermentation hydrogen production. These findings are of special interest for a large scale production of biohydrogen as the raw material is renewable, no energy input is required for the substrate pretreatment, in addition to the environmental benefits.

3.6. References

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CHAPTER 4

Semi-pilot scale production of hydrogen from Organic Fraction of Solid Municipal Waste and electricity generation from process effluents

4.1. Abstract

The production of hydrogen from Organic Fraction of Solid Municipal Waste (OFSMW) was studied on a semi-pilot scale. The potential of generating electricity using the process effluents was further assessed using a two-chambered Microbial Fuel Cell. A maximum hydrogen fraction of 46.7% and hydrogen yield of 246.93 ml H_2 g^{-1} Total Volatile Solids was obtained at optimum operational setpoints of 7.9, 30.29 ^{o}C and 60 h for pH, temperature and Hydraulic Retention Time (HRT) respectively. A maximum electrical power density of 0.21 Wm^{-2} (0.74 Am^{-2}) was recorded at 500 Ω and the chemical oxygen demand (COD) removal efficiency of 50.1% was achieved from the process. The process economics of energy generation from organic wastes could be significantly improved by integrating a two-stage process of fermentative hydrogen production and electricity generation.

***Keywords*:** Fermentative hydrogen production, Organic Fraction of Solid Municipal Waste (OFSMW), Electricity generation, Microbial Fuel Cell (MFC), Bioenergy

4.2. Introduction

The effects of climate change, increased global demands for oil and natural gas are intensifying the search for alternatives to fossil fuels (Hallenbeck and Ghosh, 2009). Hydrogen gas is an attractive future energy carrier due to its clean, efficient and renewable properties (Kapdan and Kargi, 2009) and can be generated from various organic wastes. The feasibility of hydrogen production in dark fermentation with the Organic Fraction of Solid Municipal Waste (OFSMW) in laboratory scale experiments has been reported in various studies with yields of 76 ml g^{-1} VS (Dong *et al.*, 2009), 122.9 ml g^{-1} COD (Kim *et al.*, 2004) and 134 ml g^{-1} COD (Zhou *et al.*, 2012). These were achieved under different optimal flask operational conditions. The industrial production of hydrogen from these wastes requires further understanding of the process dynamics at semi-pilot or large scale.

OFSMW is highly considered as substrate of choice for hydrogen production partly due to waste disposal problems and also its rich content of carbohydrate, biodegradability, and a high hydrogen potential (Pan *et al.*, 2008b; Shin *et al.*, 2004). South Africa generated 7.88 Mt of organic waste in 2011, and only 35% of these were recycled. The rest were mostly burnt or disposed on landfills (DEA, 2012). Hydrogen production from these waste materials will not only contribute to sustainable energy but also assists to alleviate environmental hazards.

Hydrogen production from organic waste materials is more efficient, but much of the organic matter remains in solution. Current fermentation processes can only produce 2–3 mol H_2 mol^{-1} glucose, and results in 80-90% of initial chemical oxygen demand (COD) remaining in solution in the form of various volatile organic acids and solvents (Liu *et al.*, 2010). To improve the economics of hydrogen production from substrates, additional processes are therefore needed to recover the remaining energy (Liu *et al.*, 2005). Recently, there has been an upsurge of interest in using MFC technology for harnessing electricity generation from wastewaters and organic wastes while facilitating complete energy recovery and reducing the waste treatment costs (Cheng and Logan, 2007; Mohan *et al.*, 2008). MFCs are biochemical catalyzed systems that generates electrical energy through the oxidation of biodegradable organic matter in the presence of fermentative bacteria (Logan, 2004). The bacteria present in the anode chamber of fuel cell generate electrons and protons, and the potential between the respiratory system and electron acceptor generates electricity. Hence, bacterial energy is directly converted to electrical energy. Protons migrate through a proton exchange membrane from anode to cathode (Mohan *et al.*, 2008). MFC processes have been reported for an effective energy recovering from wastewater (Cheng *et al.*, 2006; Liu *et al.*, 2010; Wang *et al.*, 2013).

This work describes a semi-pilot scale production of hydrogen from OFSMW, then investigates the electricity generation potential from the process effluents using MFC.

4.3. Materials and methods

4.3.1. Hydrogen production in a semi-pilot scale reactor

4.3.1.1. Inoculum development

The hydrogen-producing mixed consortia was obtained from the anaerobic sludge collected from the Darvill wastewater treatment plant, Pietermaritzburg, South Africa. The sludge was heated at

100 °C for 30 minutes to deactivate the methanogenic bacteria, thus enabling the survival of hydrogen producing endospore-forming clostridia which were confirmed in our previous studies (unpublished results).

4.3.1.2. Substrate pre-treatment

Organic wastes were collected from food stores in Pietermaritzburg, South Africa and the OFSMW was simulated according to the method of Gomez *et al.* (2006). It was made up of 10% apple, 10% orange, 35% cabbage, 35% potatoes, 8% bread, and 2% paper. The total volatile solids content of OFSMW was determined according to Equation (1).

$$\text{Total Volatile Solids} = \frac{\text{Weight of dried waste} - \text{Weight of ash}}{\text{Weight of dried waste}} \times 100\% \quad (1)$$

4.3.1.3. Intermediate fermentation process phase

Prior to the pilot-scale process, an intermediate fermentation stage was carried out in a 1000 ml modified Erlenmeyer flask reactor, inoculated with 50 ml of pre-treated sludge. The reactor was fed with OFSMW at concentration of 40.45 gl^{-1}, supplemented with inorganic salts (in gl^{-1}): NH_4Cl 0.5, KH_2PO_4 0.25, K_2HPO_4 0.25, $MgCl_2.6H_2O$ 0.3, $FeCl_3$ 0.025, $ZnCl_2$ 0.0115, $CuCl_2$ 0.0105, $CaCl_2$ 0.005 and $MnCl_2$ 0.015. The working volume was made up to 500 ml with distilled water. Anaerobiosis was created by flushing the reactor with nitrogen gas for 3 minutes. The setpoints of initial pH, temperature and stirring speed were 7.9, 30.29 ^oC and 1.66 s^{-1} respectively and the process was carried out for 60 h.

4.3.1.4. Fermentation process

The semi-pilot hydrogen fermentation process was conducted in 10 L bioreactor (Labfors Infors HT bioreactor, Switzerland). Prior to use, the reactor was sterilized by autoclaving at 121 ^oC for 15 minutes. It was fed with 4500 ml medium of OFSMW and inorganic salts stated above, followed by inoculation at 10% with the previous 60 h intermediate culture. The temperature was controlled at 30.29 ^oC and the stirring speed was maintained at 1.66 s^{-1}. The initial pH of the reactor was adjusted at 7.9 with no further pH control. Anaerobiosis was created by flushing the reactor with nitrogen gas for 10 minutes through the gas sparger. The Labfors Infors HT bioreactor used for biohydrogen fermentation processes is shown in Figure 4.1.

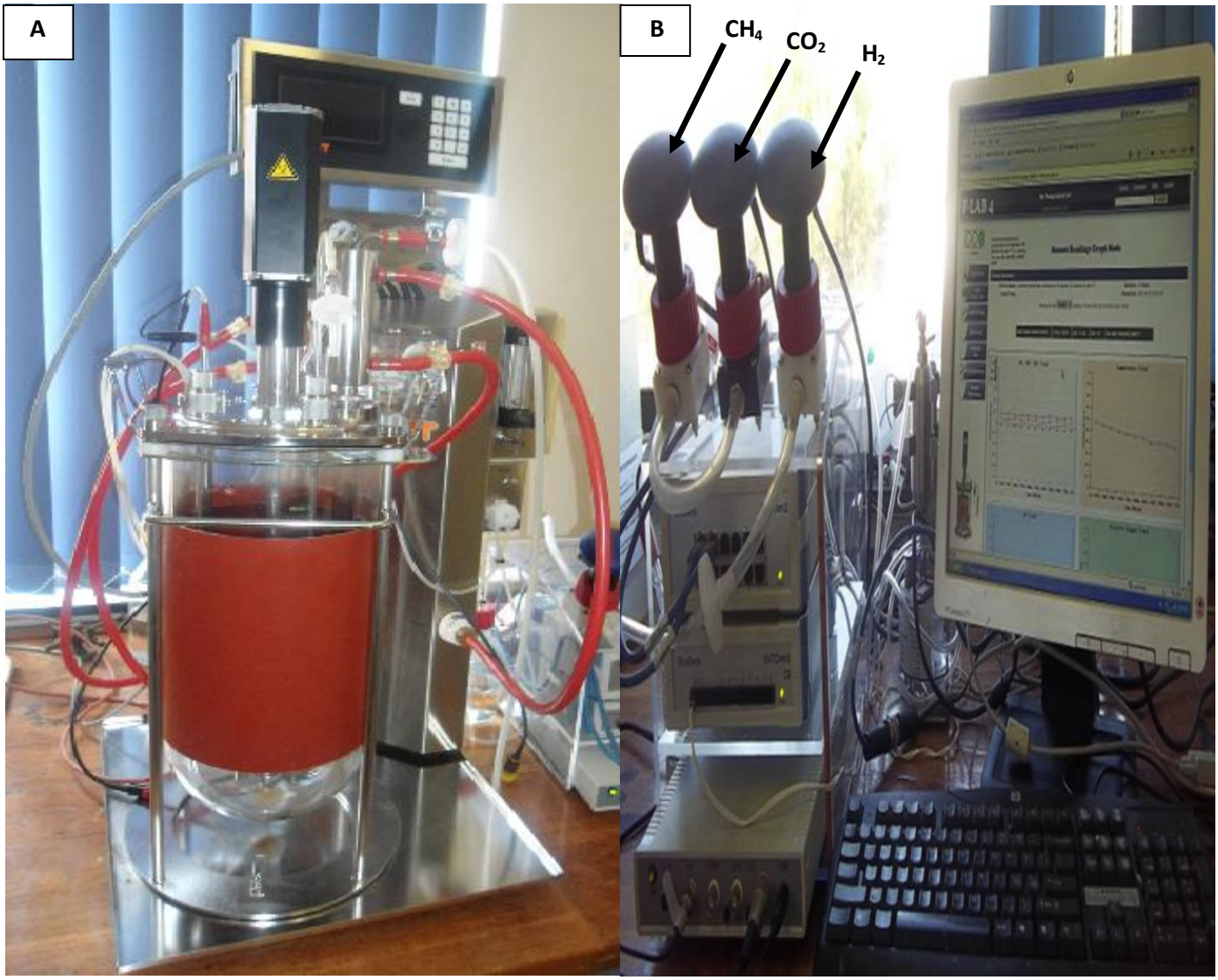


Figure 4.1: Labfors Infors HT benchtop 10 L bioreactor used for biohydrogen fermentation processes **(A)**, and **(B)** real-time monitoring station using F-Lab biogas software connected to sensors of H_2, CO_2 and CH_4 respectively.

4.3.1.5. Process monitoring and analysis

The changes in the volume fractions of hydrogen and carbon dioxide of the evolving gas were continuously monitored using the F-Lab biogas software previously described (Gueguim Kana *et al.*, 2013), running at 1 minute sampling frequency and using the BCP-H_2, and BCP-CO_2 sensors (Bluesens GmbH, Germany). The measuring principle of the gas sensors was based on thermal conductivity detector and infrared technology, all with pressure compensation. The cumulative volume of these biogas was recursively software computed using their fractions in the evolving gas and the gas volume at each sampling interval according to Equation (2).

$$V_{H,i} = V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V_H (C_{H,i} - C_{H,i-1}) \quad (2)$$

Where $V_{H,i}$ and $V_{H,i-1}$ are cumulative hydrogen gas volume at the current (i) and previous (i-1) time intervals, $V_{G,i}$ and $V_{G,i-1}$ the total biogas volumes in the current and previous time intervals, $C_{H,i}$ and $C_{H,i-1}$ the fraction of hydrogen gas in the headspace of the reactor in the current and previous time intervals, and V_H the total volume of headspace in the reactor (Chong *et al.*, 2009a).

The pH was monitored with a pH sensor (Mettler Toledo GmbH 405-DPAS-SC-K8S/325, Germany). Volatile fatty acids analysis was conducted at Nutrilab (Pretoria, South Africa). Samples were analyzed using gas chromatography (Varian 3700 FID GC, USA), equipped with SP2330 column (2 m × 3 mm) as previously described by Webb (1994). Nitrogen was used as a carrier gas at flow rate of 30 ml/min.

4.3.1.6. Isolation and morphology characteristics of hydrogen-producing bacteria

Bioreactor samples from the exponential phase of hydrogen fermentation were transferred into sterile 2 ml microcentrifuge tubes and stored at -20 °C. Tenfold serial dilutions of samples were prepared by transferring 1 ml aliquot to 9 ml ringers' solution in a range of 10^{-2} to 10^{-6}, 1 ml of appropriate dilutions was pour plated on Differential Reinforced Clostridial Agar (DRCA) and Nutrient Agar (NA) plates. Plates were grown in anaerobic jars (Oxoid Ltd, UK) at 30 °C for 72 hours. The morphology of hydrogen-producing bacteria was confirmed by gram reaction and cells were viewed under light microscope (Olympus Ax70, Japan) at 1000x magnification.

4.3.1.7. DNA extraction and 16S rRNA gene sequence analysis

Single colonies were randomly selected from the plates and suspended in 50 µl of Millipore water (Whitehead Scientific, Durban, South Africa). DNA of pure cultures was extracted using a freeze-thaw method involving heating at 100 °C for 10 minutes followed by freezing in liquid nitrogen for 10 minutes. Samples were centrifuged at 14000 g for 10 minutes and 5 µl of the supernatant was used in PCR analysis. PCR was performed using a G-STORM thermal cycler (Vacutec, Johannesburg, South Africa) in 25 µl reaction volumes containing 0.5 µl of each primer, 5 µl of DNA, 12.5 µl of 2X KAPA2G Robust HotStart ReadyMix (Kapa Biosystems, Cape Town, South Africa) and 6.5 µl Millipore water. The primers used were BacF universal primers (5'-GGGAAACCGGGGCTAATACCGGAT-3'; forward primer) and R1378 (5'-CGGTGTGTACAAGGCCCGGGAACG-3'; reverse primer) targeting universal-consensus 16S rDNA fragment (Garbeva *et al.,* 2003).

The amplification consisted of a DNA denaturing step at 95 °C for 3 minutes, followed by 35 cycles of 94 °C for 1 minute, 65 °C for 90 seconds, 72 °C for 2 minutes, final extension at 72 °C for 10 minutes. The amplification products (1500 bp) were analyzed by electrophoresis at 100 V for 30 minutes in 1% (w/v) agarose gel and visualized under UV light after being stained with SYBR Green dye. The products were sequenced at CAF DNA Sequencing Unit, Stellenbosch, South Africa using an ABI 3130xl Genetic Analyzer. The obtained 16S rRNA sequence was compared with the database sequence available in the National Center for Biotechnology Information (NCBI). The sequences were aligned using Clustal W and a phylogenetic tree was constructed from these aligned sequences by neighbour-joining method using MEGA 5 software (Tamura *et al.,* 2011).

4.3.2. Electricity generation from process effluent using MFC

4.3.2.1. MFC structure and design

The MFC was constructed as described by Khan *et al.* (2012) on a two-chambered design using glass material. The anodic and cathodic compartments were provided with inlets and sampling ports. A salt bridge made up of glass tube was used to connect the two chambers (length = 0.05 m, diameter = 0.012 m), and consisted of 10% agar, 5% KCl and 5% NaCl. The electrodes were made up of graphite rod (1.48 m^2 cross section), positioned at a distance of 0.05 m on either side of the salt bridge with equal projected surface areas of 2.19 m^2. Anaerobic conditions in the

anode were achieved by sealing the flask with a rubber stopper. The cathode was operated under aerobic conditions. Prior to use, the electrodes were sterilized with 70% ethanol. The schematic diagram of MFC design is shown in Figure 4.2.

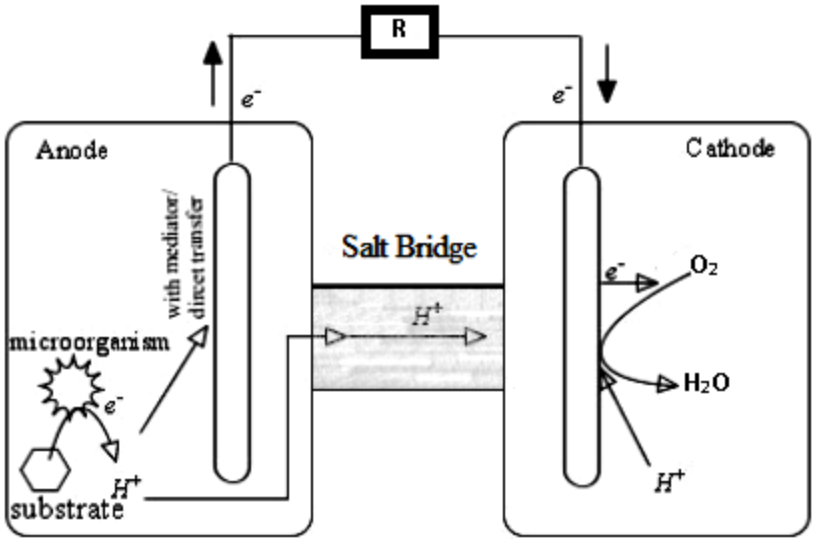


Figure 4.2: Schematic diagram of the Microbial Fuel Cell used.

4.3.2.2. MFC operation

The anodic chamber was fed with 630 ml of effluents from the semi-pilot scale fermentation bioreactor, and then inoculated with 70 ml of untreated sludge. Methylene blue (0.05 gl^{-1}) was used as a mediator in the anodic chamber. The cathodic compartment (700 ml working volume) was filled with 5% NaCl; air was continuously bubbled into the cathode for sufficient supply of dissolved oxygen within the medium. The pH of the effluent was adjusted to 7 using 10^3 mol m^{-3} sodium hydroxide. The anodic chamber was flushed with nitrogen gas (3 minutes) to create anaerobiosis. The outlet port of the anodic reactor was connected to a water displacement cylinder to collect the biogas (hydrogen, methane and carbon dioxide) produced during electricity generation. The experiment was conducted in duplicates at constant temperature (30 ^oC) using a water bath.

4.3.2.3. MFC analytical procedure and calculations

The voltage (V) in the MFC system was monitored and recorded every 3 h intervals using a digital multimeter (MDI10 Digital Multimeter, Major Tech, South Africa). For polarization, the

voltage was recorded at varied external resistance from 75 to 2000 Ω connected for 15 minutes. The current (I), power (P), power density (PD), and current density (CD) were calculated according to Mohan *et al.* (2009). PD and CD were normalized to the anode surface area (2.19 m^2). The pH of anodic chamber was recorded daily using a bench top pH meter (Lasec, South Africa). The concomitant biogas produced during electricity generation was estimated according to Equation (2). The performance of MFC was also evaluated by assessing the COD removal efficiency during operation according to Equation (3). COD analysis was performed according to the standard methods (APHA, 1998).

$$\%\ \text{removal effeciency of COD} = \frac{COD_i - COD_f}{COD_i} \times 100\% \quad (3)$$

Where COD_i and COD_f represents the influent and effluent COD concentrations (gl^{-1}) respectively.

4.4. Results and discussion

4.4.1. Lag phase of hydrogen production

The volume fractions of hydrogen and carbon dioxide were continuously monitored. As shown in Figure 4.3 A, the hydrogen production started after 4 h of fermentation. This short lag phase is due to the rich carbohydrate content of the OFSMW and its various organic matter composition which make it easily accessible to mixed microbial cultures as earlier reported by Zhou *et al.* (2012). This substrate primarily consists of kitchen type of waste with low lignin content which ranges from 0.9 to 12% (Komilis and Ham, 2003) as compared to agricultural waste residues which have a complex polymer structure. The duration of lag phase can also be affected by the operational parameters such as pH and temperature. Comparative studies showed that the lag phase times are shorter at alkaline and mesophilic conditions compared to acidic and thermophilic conditions. This is attributed to the fact that the cytoplasm of bacterial species has a higher pH and its metabolism is not disrupted by alkaline conditions (O'Sullivan and Condon, 1999). However, lag phase times are longer under acidic conditions due to disruption of cell's metabolism. Therefore bacteria have to induce acid tolerance response mechanism (Cotter and Hill, 2003). It has been reported that the activity of hydrogenase enzyme is inhibited by the low pH (Khanal *et al.*, 2004). In laboratory flask experiments, lag phase times of 2.4, 4.8 and 14 h have been reported with substrate of lettuce, potato and rice respectively (Dong *et al.*, 2009).

These substrates are easily hydrolyzed by hydrogen producing bacteria due to their biodegradable nature. In contrast, Lee and Chung (2010) reported a relatively longer lag phase time of 24 h in a two-stage pilot scale process with 150 L working volume of hydrogen production using food wastes under near similar operational conditions, and this was attributed to the nature and composition of substrate. In addition, factors such as the reactor configuration and volume size affect the partial pressure and heat transfer within the reactor in pilot scale processes and hence the lag phase duration for hydrogen fermentation process is affected. A longer lag phase times observed in pilot scale studies from organic wastes may be due to practical engineering aspects such as the size and design of the reactor which affects parameters such as mixing, heat transfer and partial pressure in large scale fermentation processes. The results obtained in this study with a lag phase time of 4 h are in line with reported findings of hydrogen production of 0.1 to 3.6 h (Shin *et al.*, 2004) and of 0.05 to 4.9 h (Pan *et al.*, 2008b) from food wastes in laboratory flask experiments at mesophilic conditions.

4.4.2. Exponential and peak production phase of hydrogen

The exponential phase of hydrogen production spanned from the process time of 4 h to about 32 h reaching a maximum hydrogen fraction of 46.5% and a cumulative hydrogen volume of 3118 ml (Figures 4.3 A and B). Zhou *et al.* (2012) reported an exponential growth phase of 21.2 h (8.8 h to 30 h) for anaerobic co-digestion of food waste and wastewater for hydrogen production in laboratory batch flask experiments. Hydrogen is produced during the exponential growth phase of clostridia in acidogenic process (Chong *et al.*, 2009b). During this process, *Clostridium* species which are either proteolytic or saccharolytic organisms hydrolyze the substrate via acetate or butyrate fermentation reaction to produce hydrogen (Khanal *et al.*, 2004). Spore germination and hydrogenase enzyme activation in hydrogen producing bacteria are observed during this process stage (Hawkes *et al.*, 2002). These have been reported as the most important factors in the overall hydrogen fermentation process (Dabrock *et al.*, 1992; Ueno *et al.*, 1996). The morphology of the prevailing hydrogen producing bacteria was observed using light microscope during this phase of fermentation (Figure 4.4). Microbial population consisting predominantly of rod-shaped cells confirmed the presence of hydrogen producing clostridia within the bioreactor. Microbial community analysis of various hydrogen producing activated sludge systems showed that *Clostridium* species are dominant active hydrogen producers (Wang

and Wan, 2008). Their presence is reported to be more than 60% of total bacterial populations after pre-treatments (Pan *et al.*, 2008a). Their dominance is possibly enhanced by the resistance of endospores (Fang *et al.*, 2006).

The fermentation process showed a peak of hydrogen fraction of 46.7% with a cumulative hydrogen volume of 3139 ml at 33 h and lasted for 1 h. The duration of a steady peak hydrogen fraction depends on the substrate type and process conditions. For instance, Dong *et al.* (2009) reported peak durations of 1, 1 and 3 h for potato, rice and lettuce respectively in laboratory flask processes at pH 5.5 and 37 ^{o}C. Whereas Lay *et al.* (1999) observed a peak duration of 6 h in hydrogen production process from organic municipal waste under similar operational conditions. The reported peak of hydrogen fraction in semi-pilot scale varies with reactor size, process time and substrate used. For example, Lin *et al.* (2011) using a 400 L bioreactor operated for 65 days obtained a peak in hydrogen fraction of 37.8% using sucrose medium and Chang *et al.* (2011) using a 12 L bioreactor operated for 95 days obtained a peak value of 40.4% on molasses. With regard to process yield at semi-pilot scales, values of 1.04 mol H_2 mol^{-1} sucrose at 400 L (Lin *et al.*, 2011), 2.91 mol H_2 mol^{-1} hexose at 20 L (Masset *et al.*, 2012) and 1.40 mol H_2 mol^{-1} glucose at 12 L (Chang *et al.*, 2011) have been reported. These observations point to the scale-dependent hydrogen production efficiency which might be due to traditional fermentation scale up challenges.

4.4.3. Process decline phase

A decrease in hydrogen fraction was observed from process time of 34 h to 64 h and reached a minimum hydrogen fraction value of 6.9% (Figure 4.3 A). This can be attributed to the switch of fermentation process from acidogenic to solventogenic process as earlier reported by Khanal *et al.* (2004). Thus the change in process intermediates products from acetate, butyrate to acetone, butanol and ethanol or the acidogenic–solventogenic transition led to inhibition of hydrogen production. Hydrogen consuming bacteria such as homoacetogens can also pose a threat to hydrogen producers because these are versatile group of bacteria, strictly anaerobe, fast growing and endospore-forming organisms (Pan *et al.*, 2008a). These bacterial species grow chemolithoautotrophically on hydrogen and carbon dioxide, producing acetate at higher hydrogen thresholds than methanogens or sulfate-reducing bacteria (Khanal *et al.*, 2004). They have higher growth rates than other fermentative bacteria due to energy conservation from a

combination of substrate-level phosphorylation and sodium-based chemiosmotic mechanisms (Muller, 2003).

4.4.4. Carbon dioxide evolution

The carbon dioxide production started from process time of 4 h and reached a maximum fraction of 28.4% and a cumulative volume of 1435 ml at 14 h (Figure 4.3 A). During this process time, a very high correlation (0.99) was observed between hydrogen and carbon dioxide evolution. This could be attributed to the acetate and butyrate fermentation pathways that generate 2 mol CO_2 mol^{-1} glucose. However, a steady carbon dioxide fraction of 28.4% was observed from 15 h to 24 h. It is likely that acetate fermentation was thermodynamically favoured at this stage since it has a high theoretical yield of hydrogen (4 mol H_2 mol^{-1} glucose). Acetate and butyrate reactions are formed during dark fermentation processes but their ratio varies with growth conditions (Thauer *et al.*, 1977). Earlier studies by Van Andel *et al.* (1985) showed that decreasing the partial pressure of hydrogen resulted in an increase in acetate/butyrate ratio and in turn enhances the hydrogen production.

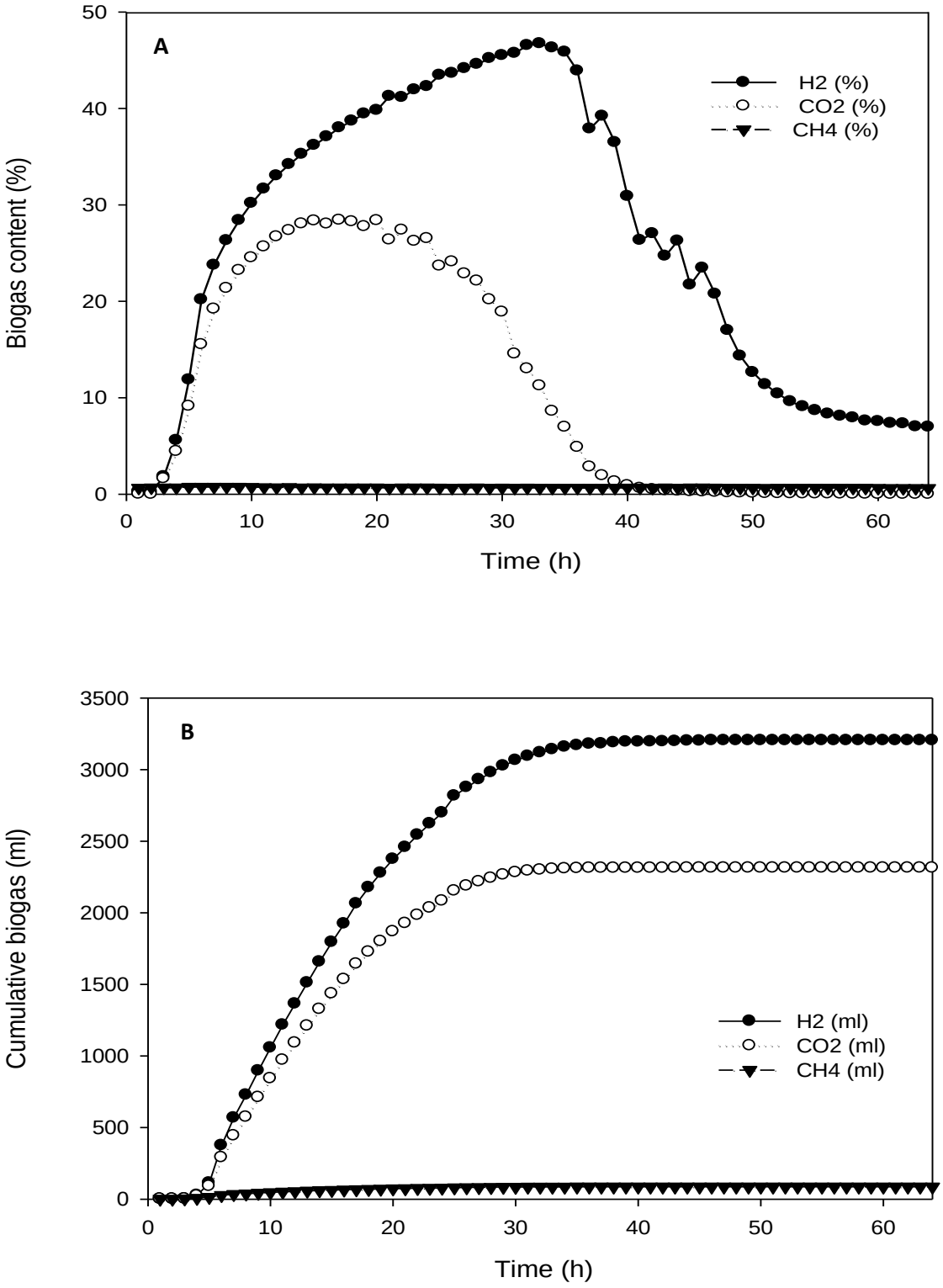


Figure 4.3: Evolution of biogas fractions of hydrogen, methane and carbon dioxide **(A)**, and **(B)** the trends in cumulative biogas during a semi-pilot continuous monitoring process.

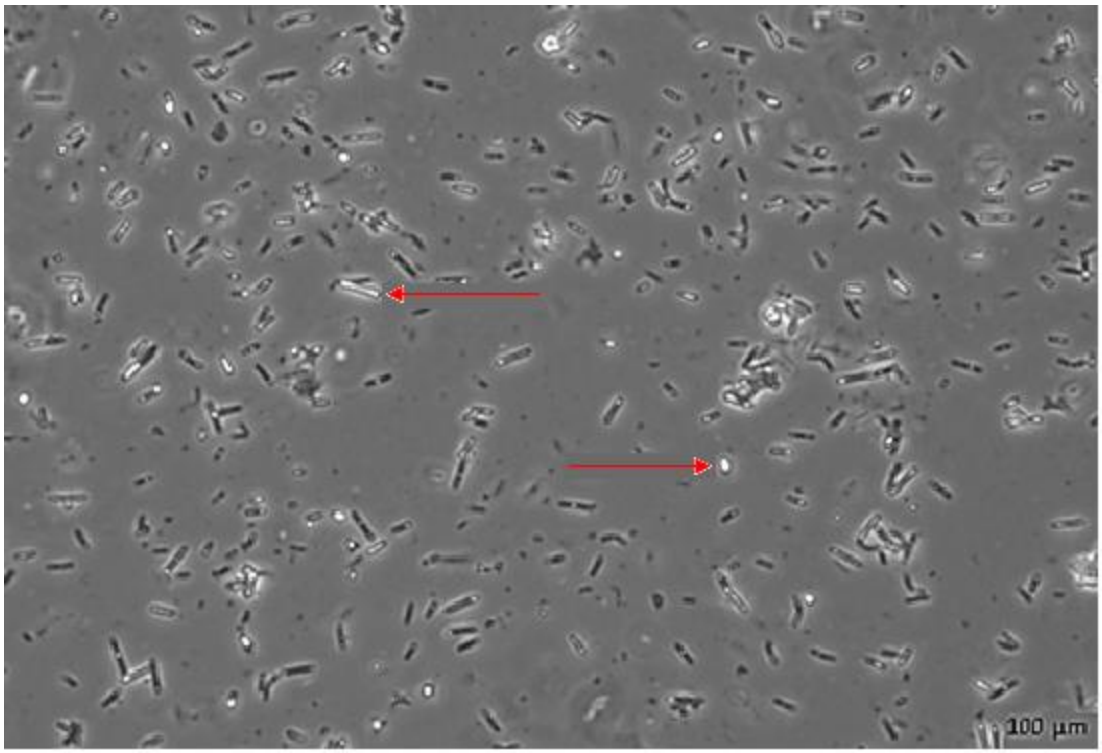


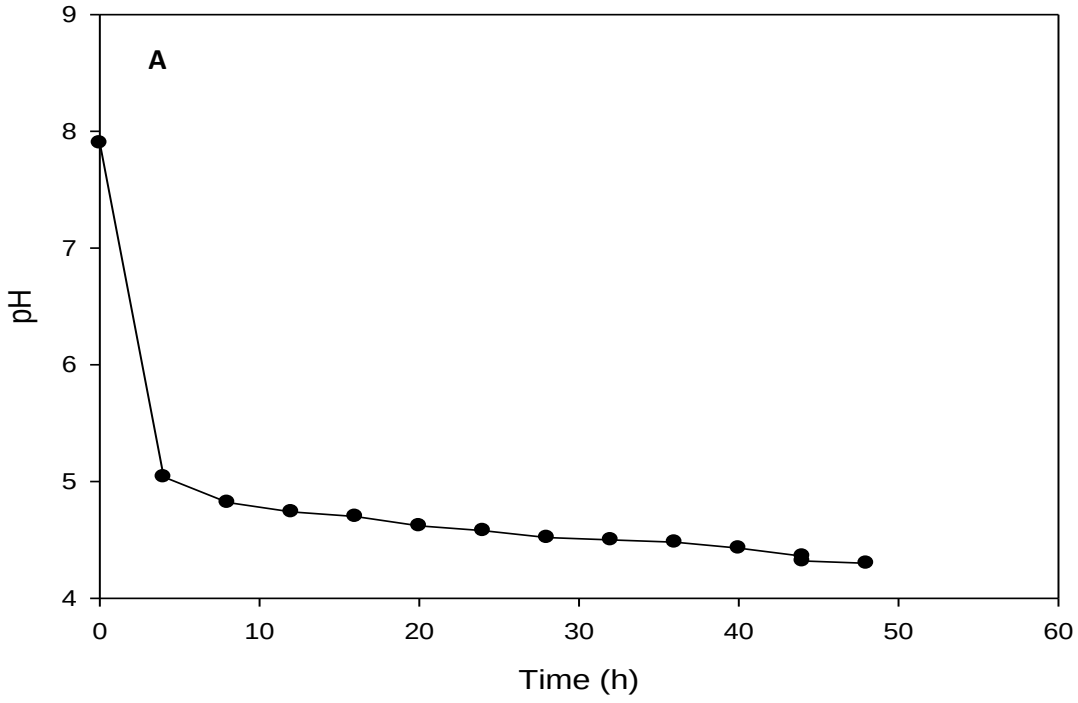
Figure 4.4: Morphology of hydrogen-producing bacteria. Sporulating rod-shaped cells are indicated with an arrow.

4.4.5. pH evolution during semi-pilot fermentation process

A decrease in pH from 7.9 to 5.04 was observed during the first 4 h of hydrogen fermentation process (Figure 4.5 A). In the previous studies, we reported a pattern of a sharp drop in pH at the late lag phase which was an early indicator for the onset of the log phase in dark fermentation process monitoring (Gueguim Kana *et al.*, 2013). Hydrogen is associated with the production of volatile fatty acid (VFA) components such as acetate, butyrate and propionate (Kapdan and Kargi, 2006). The pH drop represents rapid production of VFAs within the medium (Mohan *et al.*, 2008). From the process time of 10 h to 46 h, the pH remained relatively stable within a range of 4.7 to 4.3 without the addition of a buffer. A similar observation has been reported by Zhi *et al.* (2008) for a pH decrease from 7 to a relatively constant range of 4.65 to 4.85 in a non-buffered hydrogen production system. It is likely that this relative stability might be due to a balanced uptake of protons by hydrogenases according to Equation (4).

$$2H^+ + 2e^- \longleftrightarrow H_2 \qquad (4)$$

The control of pH during hydrogen fermentation remains necessary to prevent a possible metabolic shift and to suppress the hydrogen consumers while maintaining an enriched culture of hydrogen producing bacteria. pH control is more feasible at pilot scale using dedicated sensors and actuators (Chang *et al.*, 2011; Lin *et al.*, 2011) than in water bath shake flask systems. In the later, only the initial pH value is often reported.



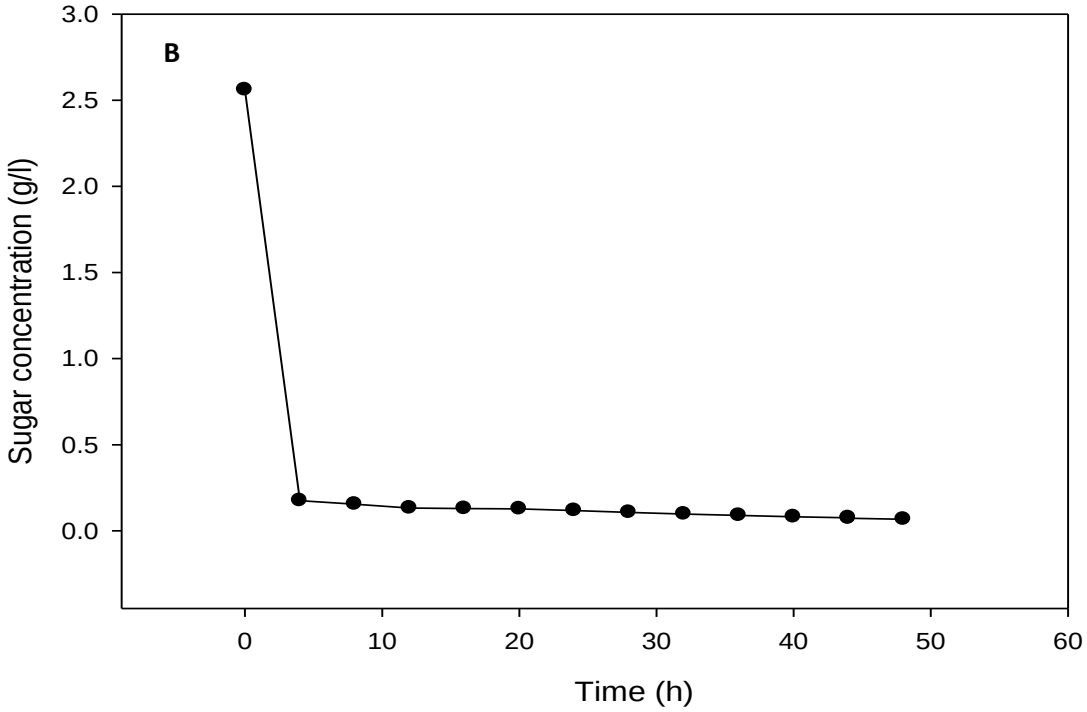


Figure 4.5: pH profile **(A)**, and **(B)** sugar degraded by microorganisms during a semi-pilot continuous monitoring dark fermentation process.

4.4.6. Production of volatile fatty acids

Dark fermentation process is associated with the production of metabolites such as acetate, butyrate, propionate, valerate and ethanol which reflect changes in metabolic pathways of hydrogen producing consortia during acidogenic–solventogenic transition. A better knowledge of such changes could improve our understanding of mechanisms of biochemical reactions involved and conditions favourable for its production when using different substrates (Prakasham *et al.,* 2009). Thus, during the course of hydrogen production process, liquid samples from the bioreactor were collected and analyzed for individual volatile fatty acids (VFAs). The VFAs detected were acetate, butyrate and propionate (Figure 4.6), and accounted for 56.37, 41.86 and 1.77% respectively during the lag phase of hydrogen production (4 h). Meanwhile acetate increased to 68.09% and butyrate decreased to 29.82% when hydrogen was produced at exponential phase (20 h). Acetate-fermentation pathway was therefore favoured in this process. During this process, there is high production of NAD^+/NADH which increases the high yields of hydrogen (Guo *et al.,* 2008; Van Ginkel *et al.,* 2005). These results are consistent with

stoichiometric relationship of Equations (5) and (6). Based on these equations, 4 mols of hydrogen are produced from acetate-pathway and 2 mols of hydrogen are produced from butyrate-fermentation pathway. Earlier studies on hydrogen production have also shown that hydrogen-producing bacteria such as *Clostridium* species form these metabolites during their exponential growth phase (Fan *et al.,* 2004; Lay *et al.,* 1999). The production of the aforementioned VFA components suggested that both these fermentation pathways occurred simultaneously during hydrogen fermentation process as reported in literature (Liu *et al.,* 2011). Wu *et al.* (2006) indicated that there might be an optimal acetate/butyrate ratio for hydrogen production but the ratio depends on hydrogen-producing bacteria and substrate used.

Acetate: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$ (5)

Butyrate: $C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$ (6)

Studies on hydrogen production processes have pointed out that metabolites such as propionate and ethanol are not suitable for its production (Hawkes *et al.,* 2007; Li and Fang, 2007). Higher acetate/butyrate ratios and lower concentrations of propionic acid reflect higher efficiency of biological hydrogen production (Chen *et al.,* 2002; Han and Shin, 2004), because thermal treatment of anaerobic sludge is predominated by spore-forming microorganisms, most of which are clostridia species, which produce hydrogen during acetic and butyric acid production. The ratio of acetate decreased to 53.07% when hydrogen reached the death phase (40 h), however butyrate remained relatively high (45.8%). These results are in correlation with previous studies of hydrogen from OFSMW and food waste. Lay *et al.* (1999) reported a high acetate and butyrate concentrations of 0.97 and 2.81 g/l respectively from OFSMW. Shin *et al.* (2004) reported an acetate and butyrate concentrations of 137 and 898 mg/l from food waste. Similar results were confirmed by Kim *et al.* (2013), they reported a high acetate/butyrate ratio and low concentration of propionate for hydrogen production from food waste.

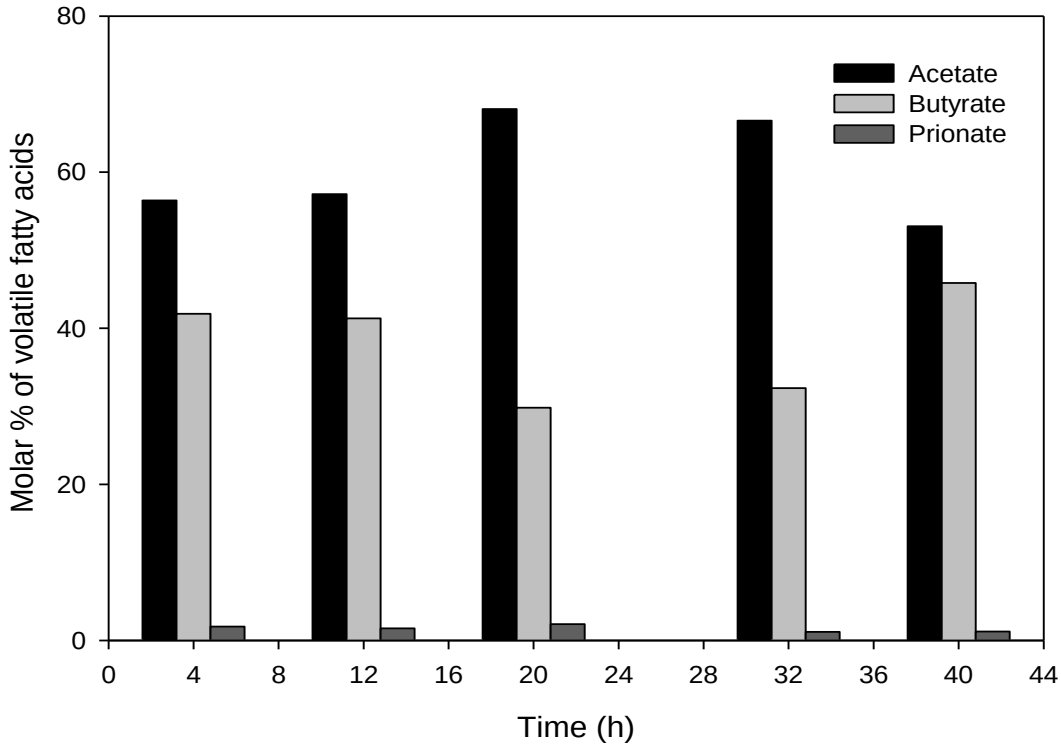


Figure 4.6: Production of volatile fatty acids during hydrogen production.

4.4.7. Isolation of hydrogen-producing bacteria

In order to identify the hydrogen-producing microbial populations within the bioreactor, cultures of hydrogen-producing bacteria were quantified on Differential Reinforced Clostridial Agar (DRCA) and Nutrient Agar (NA) respectively. DRCA was used as a selective media for enumeration of spore-forming *Clostridium* species as recommended in literature (Weenk *et al.,* 1995). Meanwhile NA was used for enrichment of diverse groups of hydrogen-producing bacteria (Kanso *et al.,* 2011). Total genomic DNA was isolated from the colony cultures using PCR; the DNA was used as a template for profiling the bacterial community using 16S rDNA gene clone libraries. The community consisted of major bands (Lanes 1-5), and less defined bands (Lanes 6-10) as shown in Figure 4.8. These results showed that DRCA was not effective for quantification of clostridia. Studies on isolation of *Clostridium* species using DRCA have shown that few *Clostridium* strains, typically those that are butyric anaerobes such as C. *butyricum* and *C. tyrobutyricum* are not readily detected using this method since they are unable to reduce sulphite sufficiently (Byrne *et al.,* 2008; Eisgruber and Reuter, 1995). Moreover,

groups of gram-negative facultative anaerobic bacteria such as *Citrobacter* sp., *Proteus* sp., and few *Salmonella* sp. are also sulphite-reducing microorganisms. As a consequence, most recent studies of hydrogen fermentation processes rely on culture independent methods for enumeration of various communities of hydrogen-producing bacteria.

In addition, the sequence obtained for the two isolates showed a high similarity of 97 and 98% (Table 4.1) to 16S rRNA gene sequences of environmental isolates identified as *Klebsiella variicola* and *Klebsiella pneumonia* respectively. These results were confirmed by phylogenetic analysis which depicted a close relationship between the isolates and *Klebsiella* sp. (Figure 4.9). The obtained results were therefore consistent with literature; *Klebsiella* and *Clostridium* species are extensively reported in studies of hydrogen-producing sludge (Chen *et al.,* 2006; Hafez *et al.,* 2010; Kim *et al.,* 2006; Liu and Fang, 2007; Saraphirom and Reungsang, 2011). The inhibition of spore-forming clostridia might have been caused by various factors such as oxygen concentration in the reactor and the selected growth media. These microorganisms are fastidious and are extremely sensitive to oxygen. Their hydrogen-producing abilities are inhibited by small traces of oxygen in the reactor (Hung *et al.,* 2007). Thus addition of reducing agents may be necessary for ensuring stable cell growth and hydrogen production. Quantification of these microorganisms has often relied on culture independent approaches as mentioned earlier.

Abreu *et al.* (2012) conducted a microbial community analysis in hydrogen-producing reactor at thermophilic conditions (70 °C). Clones corresponding to DGGE bands present in reactor sludge exhibited highest sequence identity with *Klebsiella* sp. (99%), *Thermoanaaerobacterim* sp. (≥99%) and *Bacillus* sp. (99%). Koskinen *et al.* (2008) identified *Klebsiella oxytoca* (97.4%) as one of the dominant organisms in hydrogen-producing sludge at thermophilic conditions (58 °C) using DGGE. In another study, Masilela (2011) reported an isolates having a sequence identity of 100% with *Klebsiella* sp. for bioreactor operated at 65 °C. These results suggested that *Klebsiella* species can tolerate high temperatures. They are gram negative, facultative anaerobes and rod-shaped bacteria and are found in various habitats such as surface water, sewage sludge, soils and plants, as well as mucosal surfaces of mammals (Brisse and Verhoef, 2001). Five groups of *Klebsiella* species have been reported, these includes *K. pneumoniae* (with its three subspecies), *K. oxytoca*, *Klebsiella planticola*, *Klebsiella terrigena* and *Klebsiella mobilis* (also known as *Enterobacter aerogenes* (Brisse and Verhoef, 2001).

The presence of facultative anaerobes such as *Klebsiella* sp. plays a significant role in suppressing the oxygen in the medium, creating anaerobic conditions suitable for hydrogen production. Furthermore studies shows that *Klebsiella pneumonia* contains $NADP^+$ dependent Ni/Fe type hydrogenase (Schut and Adams, 2009), this enzyme is responsible for hydrogen production in these microbial consortia.

Most studies of hydrogen fermentation processes applied the 16S rRNA PCR-DGGE analysis for identification of hydrogen-producing microorganisms in various hydrogen-producing reactors (Fang *et al.,* 2002; Hung *et al.,* 2007; Kim *et al.,* 2006; Wang *et al.,* 2007). This approach is based on the separation of PCR-amplified same length fragments of specific genes (Hung *et al.,* 2007). However this method has its own limitations such that some bacterial strains cannot be detected especially those with low intensity (Wang *et al.,* 2007). Hung *et al.* (2008) proposed the use of in situ detection methods such as fluorescence-labelled, rRNA-targeted oligonucleotide probes for microscopic identification of hydrogen-producing bacteria in order to improve the results.

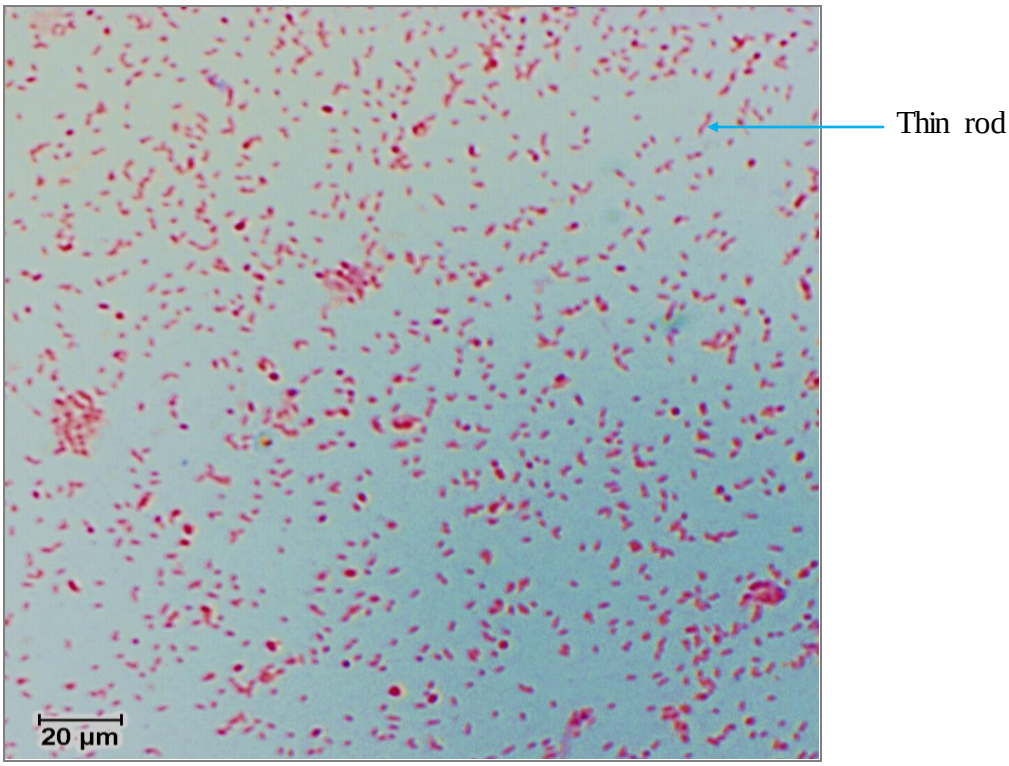


Figure 4.7: Gram stain image showing the morphology of hydrogen producing *Klebsiella* sp..

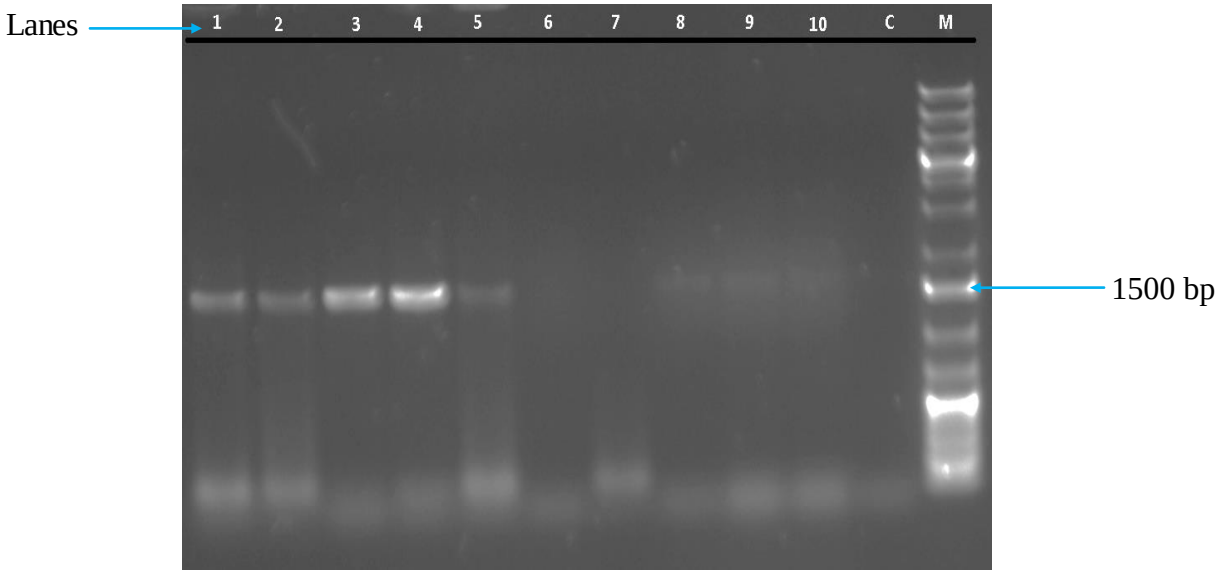


Figure 4.8: PCR profile of hydrogen producing bacteria (Lanes 1-5 correspond to genomic DNA of bacteria grown in NA, Lanes 6-10 correspond to genomic DNA of bacteria grown in DRCA, C-Control). The GeneRuler™ 1kb DNA Ladder (M) was used on 1% agarose gel to determine the size of the isolated DNA fragments (1500 bp).

Table 4.1: Affiliation of isolates to published species using 16S rRNA sequence.

		NCBI blast results		
Isolates	**Organisms affiliation**	**Query cover (%)**	**Accession no.**	**Identity (%)**
1	*Klebsiella variicola*	94	KF358449.1	97
1	*Klebsiella variicola*	94	KF224905.1	97
2	*Klebsiella pneumonia*	98	KF530729.1	98
2	*Klebsiella pneumonia*	98	KC876640.1	98

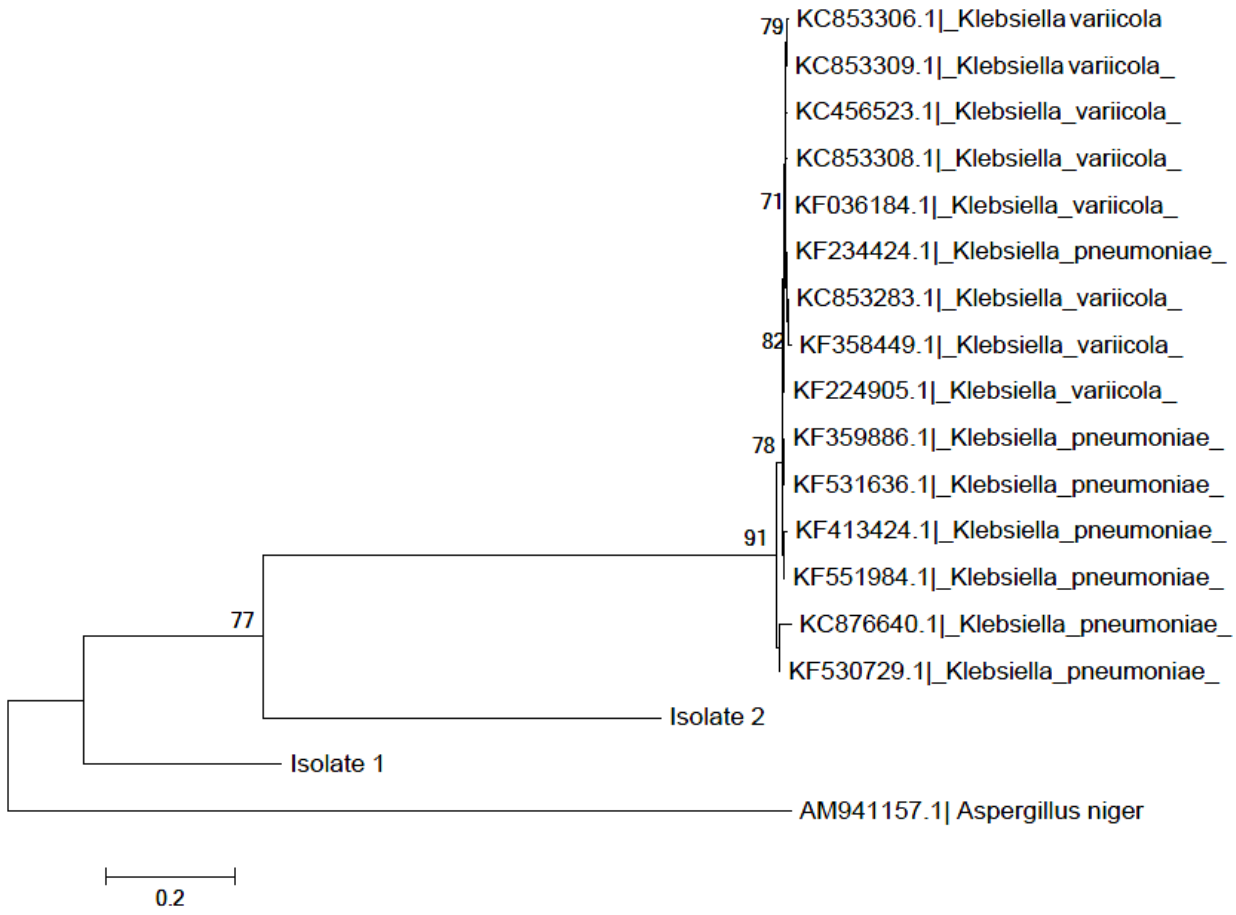


Figure 4.9: Phylogenetic tree resulting from neighbour-joining analysis of 16S rRNA sequences of the two isolates and published sequences of hydrogen producing bacteria. The numbers at the branch nodes are bootstrap values (per 1000 trials). The scale bar indicates 0.2 substitutions per site. Bootstrap values less than 70% are not shown on the tree. *Aspergillus niger* was used as an outgroup.

4.4.8. Electricity generation using process effluent

Due to the traditional low yield of hydrogen generation on dark fermentation processes, the organic substrates in the effluent are not fully metabolized. A second bioprocess stage was adopted for further energy extraction using MFC. In addition the anodic chamber of MFC can operate as wastewater treatment reactor. The electrogenic bacteria used the suspended organic matters in the effluent for biomass development and electron generation. A gradual increase in MFC voltage was observed from 0.05 V to a maximum open circuit voltage of 0.48 V after 60 h of operation (Figure 4.10 A). Thereafter it showed a decreasing trend suggesting exhaustion of nutrients. During the MFC operation, the evolving gas from anodic chamber was analyzed with respect to hydrogen, methane and carbon dioxide. A cumulative hydrogen production of 9.2 ml was recorded. Operationally, hydrogen utilization occurs during electricity generation as protons move to the cathodic chamber, thus the observed volume of hydrogen evolved from anodic chamber is lower than the actual volume. These observations point to a feasibility of a concomitant generation of electricity and hydrogen. Niessen *et al.* (2004) reported that hydrogen producing bacteria such as *Clostridium butyricum* and *C. beijerinckii* were capable of producing electricity from starch.

The polarization sweep obtained by applying various external resistance helps to determine the operational point of the MFC. In practice, as the applied resistance becomes lower, there is a greater electron demands, forcing the microbial consortium to increase the metabolic activities, and in so doing improve the power and COD removal efficiency. This is sustainable if it is near the point of Maximum Power Transfer (MPT); obtainable from a polarization curve. It is usual practice to operate the MFC to the left side of power density peak, and at high voltage or low current density (Mohan *et al.*, 2008). In this study the curve was obtained by plotting the calculated current density against the power density at various external resistance values. A maximum power density of 0.21 Wm^{-2} (0.74 Am^{-2}) at 500 Ω was obtained (Figure 4.10 B). It is not feasible to directly compare the power output with other MFC processes in literature due to difference in operational setpoint parameters, surface area and type of electrodes, and different microorganisms used (Pan *et al.*, 2010). The construction of the MFC and concentration of organic matter also affects the generation of power outputs (Logan, 2004). Oh and Logan (2005) observed that generation of electricity in single-chambered process was 3.5 times higher than

that achieved in two-chambered process, although a single-chambered MFC design has some challenges such as reverse polarization and low oxygen supply in the cathodic compartment. Some of the common electrogenic microbes with their associated maximum power densities are shown in Table 4.2. Electricity can be generated from diverse microorganisms particularly those microbes that are dominant in soil and wastewater samples (*Escherichia coli*, *Shewanell* species). MFCs can be operated using either pure or mixed cultures. Mixed cultures are more suitable for the use of complex substrates such as wastewater and biomass effluents, as single organisms generally metabolize quite a limited range of organic compounds (Kim *et al.*, 2007); as shown in Table 4.2, a higher power density of 5.85 Wm^{-2} was obtained using mixed cultures.

The pH measurements over time during MFC operation showed that the anolyte pH decreased gradually from 7.2 to 4.21 (Table 4.3), due to production of fermentative metabolites which changed the buffering capacity of the medium. The trend of pH change was in line with active electricity generation in MFC processes (Wang *et al.*, 2013). The reported optimum pH in anodic chamber of MFC is in the range of 6-7 (Pan *et al.*, 2010).

The MFC was also assessed on the COD removal potential of the anodic reactor. A decrease in COD concentration from 1.66 gl^{-1} to 0.83 gl^{-1} was obtained in the digesting effluent giving a COD removal efficiency of 50.1%. Butyrate and acetate which are the intermediate products of most fermentation are highly hydrolysable, and removal of 28.4-48.7% of acetate have been reported by Wang *et al.* (2013) while Liu *et al.* (2005) reported substrate removals of 98 and 99% for butyrate and acetate respectively. Cheng and Logan (2007) reported that electricity could be produced in MFCs from acetate at yields approaching 99%.

These data highlight the feasibility of a concomitant generation of hydrogen, electricity coupled with an efficient COD removal using anaerobic fermentation of OFSMW.

Table 4.2: Maximum power densities in various studies of MFCs.

Microorganism	Reactor type	Substrate used	Power density (Wm^{-2})	Reference
Digested sludge	Membrane-less MFC	Acetate	0.03	Wang *et al.* (2013)
Escherichia coli	Single-chambered	Complex medium	0.60	Zhang *et al.* (2006)
Shewanella oneidensis	Miniature reactor	Lactate	3.00	Ringeisen *et al.* (2006)
Wastewater	Two-chambered	Acetate	0.37	Oh and Logan (2005)
Anaerobic sludge	Two-chambered	Inorganic salts	0.16	Mohan *et al.* (2008)
Wastewater	Single-chambered	Glucose	0.77	Cheng *et al.* (2006)
Corynebacterium MFCO3	Single-chambered	Glucose	7.30	Liu *et al.* (2010)
Mixed cultures	Two-chambered	Glucose	5.85	Rosenbaum *et al.* (2006)

Table 4.3: Characteristics of the fermented effluent during electricity generation.

	Time (h)					
Parameter	**0**	**24**	**48**	**72**	**96**	**120**
pH	7.2	6.82	5.62	5.32	4.71	4.21
COD (gl^{-1})	1.66	–	–	–	–	0.83
Cumulative biogas (ml)						
Hydrogen	-	-	-	-	-	9.2
Carbon dioxide	-	-	-	-	-	5.2
Methane	-	-	-	-	-	3.8

−: Not available

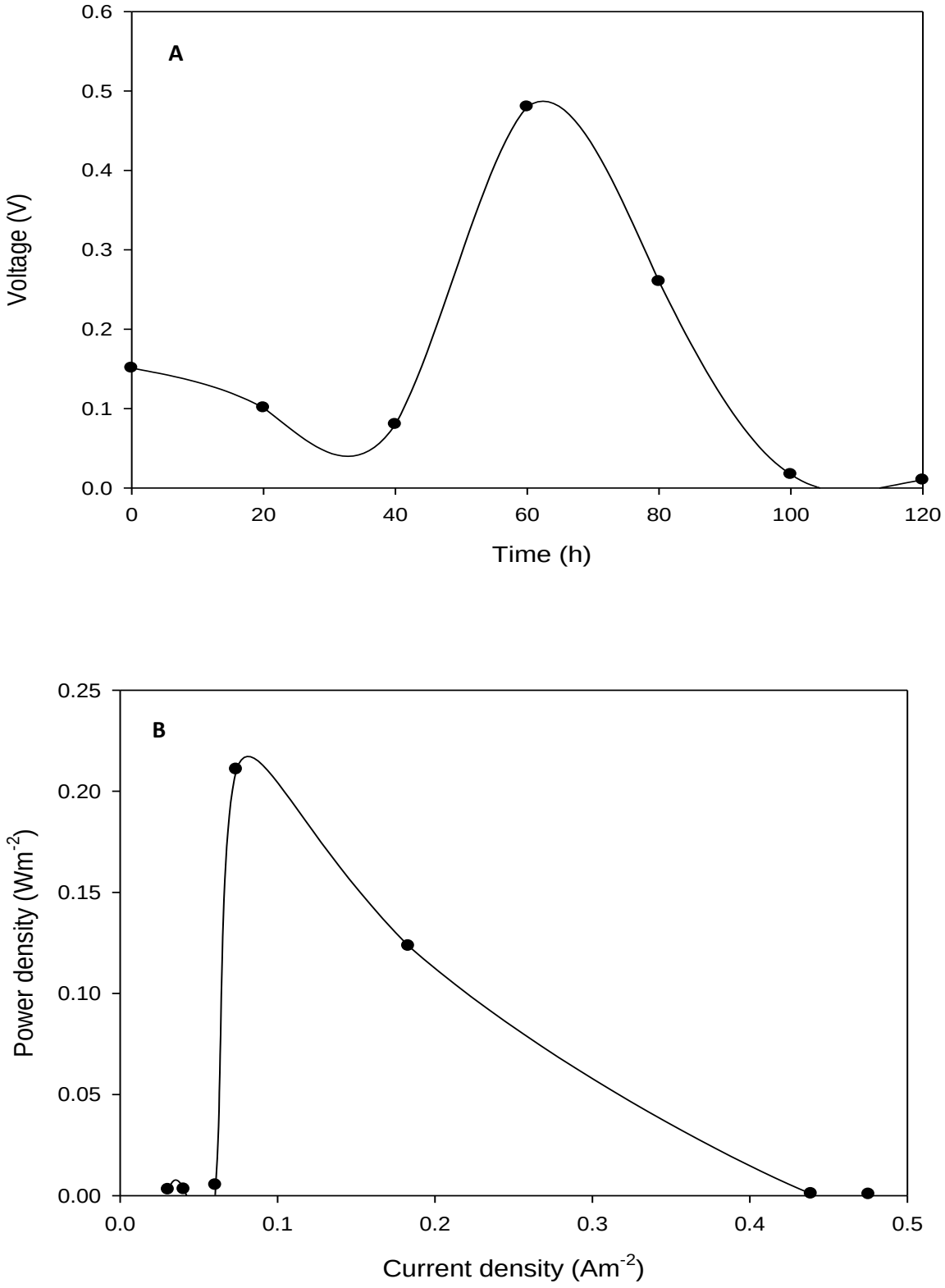


Figure 4.10: Electricity generation using fermented effluent from hydrogen producing reactor at 150 Ω **(A)**, and **(B)** Power density as a function of current density.

4.5. Conclusion

A semi-pilot scale hydrogen production process was carried out and the conversion of process effluents to electricity using Microbial Fuel Cell was assessed. The study revealed that a lag phase of 4 hours, a peak hydrogen fraction of 46.7% and yield of 246.93 ml H_2 g^{-1} Total Volatile Solids were achievable at a semi-pilot scale of dark fermentation using the organic fraction of solid municipal waste. Furthermore, electricity generation at a power density of 0.210 Wm^{-2} and a chemical oxygen demand removal efficiency of 50.1% can be obtained from the process effluents using a two chambered membrane-less Microbial Fuel Cell. These findings highlight the feasibility of hydrogen scale up on organic fraction of solid municipal waste, and a concomitant generation of electricity and COD removal from the process effluents. As the maximum theoretical yield of hydrogen production on pure glucose substrate is low (4 mol H_2 mol^{-1} glucose), further hydrogen scale up studies using the organic fraction of solid municipal waste as substrate coupled with MFC for optimum bioenergy extraction would shorten the timeline for a more environmentally friendly and sustainable hydrogen economy development.

4.6. References

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CHAPTER 5

Fermentative biohydrogen modelling and optimization research in light of miniaturized parallel bioreactors

5.1. Abstract

In the last decade, there has been an upsurge of interest to make a transition from the depleting fossil-based energy sources to renewable ones. Fermentative biohydrogen has been repeatedly flagged as a potential future alternative energy carrier in recent publications. Research towards its scale-up requires accurate and high throughput optimization data on key process parameters. This has been hampered by conflicting findings, potentially owing to research procedures and bioreactor equipments used. This study reviews the current state of fermentative biohydrogen optimization research on agricultural wastes, using miniaturized parallel bioreactors (MPBs). The monitoring and control of physico-chemical parameters on these bioreactors is discussed and the prospect of enhancing biohydrogen process development with a novel featured parallel miniaturized bioreactor is presented.

Keywords: Miniaturized Parallel Bioreactors (MPBs), Fermentation, Biohydrogen production, Bioprocess development

5.2. Introduction

Fermentative biohydrogen production is attracting increasing global attention owing to its non-polluting feature, low-cost and renewable source. Biohydrogen is a promising fuel for the future with many social, economic and environmental benefits to its advantage. It has a long-term potential to reduce the dependence on foreign oil and lower the carbon emissions from the transportation and the industrial sectors (Meher Kotay and Das, 2008). It has a high energy yield of 122 kJ/g which is 2.75 times greater than its equivalent of hydrocarbon fuels (Kapdan and Kargi, 2006), and its reaction with oxygen does not produce greenhouse gases such as carbon dioxide (CO_2). Biohydrogen production using dark fermentation is more feasible (Show *et al.*, 2007; Strobel and Nakatsukasa, 1993; Wang and Wan, 2009; Xing *et al.*, 2008; Yang *et al.*, 2006) because it generates very clean fuel hydrogen at an affordable cost. It has wide areas of application, e.g. as automobile fuel, as a source of distributed or central electricity, and for generation of thermal energy.

The achievement of higher yields is a critical research objective for the sustenance of

biohydrogen as the fuel for the future. Biohydrogen productivities of 605 $mg \cdot h^{-1} \cdot L^{-1}$ by an undefined consortium is the highest productivity that has been reported so far (Das *et al.*, 2008). But this process is still not commercially viable (Yoshida *et al.*, 2006). The maximum biohydrogen molar yield on glucose reported is 2.91 mol H_2/mol hexose (Masset *et al.*, 2012). Besides, the production of hydrogen from glucose is too expensive to support economic H_2 production.

Research towards biohydrogen scale-up requires accurate and high-throughput optimization data on key process parameters, generated from multifactorial experimentation using state-of-the-art miniaturized and paralleled bioreactors. This study reviews the current state of fermentative biohydrogen optimization research on agricultural wastes, using miniaturized parallel bioreactors (MPBs). The monitoring and control of physico-chemical parameters on these bioreactors is discussed and the prospect of enhancing biohydrogen process development with a novel featured parallel miniaturized bioreactor is presented.

5.3. Biohydrogen production from agricultural wastes

Biohydrogen is produced from a wide variety of biomass substrates, including agricultural and forestry wastes, municipal solid wastes and animal wastes and residues (Muradov and Veziroglu, 2008; Tefferi and Vardiman, 2007). Many agricultural and food industry wastes contain starch and/or cellulose which are rich in carbohydrates. The complex nature of these wastes may adversely affect their biodegradability. Starch-containing solid wastes are easier to process for carbohydrate and hydrogen gas formation (Kapdan and Kargi, 2006). There are three obstacles to the economical production of glucose from cellulose-rich biomass: (i) most biomass is quite dispersed, making its collection costly, even though its intrinsic raw material price is low; (ii) the structure of cellulosic materials, with cellulose fibrils surrounded by hemicelluloses and then lignin, is difficult to penetrate; and (iii) the cellulose chain is difficult to break down to glucose and other sugars either chemically or enzymatically. The production of biohydrogen from crop waste biomass is limited by the hydrolytic activity of the microorganisms involved in the biological attack of the heterogeneous and microcrystalline structure of lignocellulosic component, and in the decomposition of cellulose-like compounds to soluble sugars.

Zhang *et al.* (2007) reported a yield of biohydrogen of 57 $mL \cdot g^{-1}$ when cornstalk was treated with sodium hydroxide (0.5 % NaOH); this value was 19-fold higher than the yield obtained from untreated materials (3 $mL \cdot g^{-1}$). Furthermore, the authors investigated the production of

biohydrogen from cornstalk waste with mixed pre-treatments of acid (0.2 % HCl) and heat, with a maximum yield of 150 $mL \cdot g^{-1}$, or a 50 times increase as compared to the initial value, thus proving the efficiency of both acid and base pre-treatment methods. Wang and Jin (2009) optimized fermentative biohydrogen production, using sugarcane molasses. The maximum biohydrogen yield obtained was 1.85 mol H_2/mol hexose; corresponding to a biohydrogen rate of 17.38 $mmol \cdot h^{-1} \cdot L^{-1}$. It was observed from these results that organic substrates rich in carbohydrate and protein content are suitable for maximum biohydrogen production. Kongjan *et al.* (2009) investigated the optimization of biohydrogen production from wheat straw hydrolysate, using dark fermentation in both batch and continuously stirred tank reactors. The maximum hydrogen yield was 318.4 $mL \cdot g^{-1}$ at a hydrolysate concentration of 5 % (v/v) in a batch reactor. In the continuously stirred tank reactor, the hydrogen yield and production rate were 178.0 $mL \cdot g^{-1}$ and 184.0 $mL \cdot (day \cdot L_{reactor})^{-1}$ (operating for 3-day HRT) respectively, corresponding to 12 % of the chemical oxygen demand (COD) from sugars. In another study, Chen *et al.* (2002) enhanced biohydrogen production from untreated rice straw, using mixed cultures. The maximum cumulative hydrogen production, hydrogen production rate and hydrogen lag phase were 733 mL, 18 $mL \cdot h^{-1}$ and 45 h, respectively.

Appropriate pre-treatment steps for the raw material are often required to enhance hydrolysis. The main pre-treatments are based on mechanical, physical, chemical and biological techniques (Mtui, 2010). A mechanical shredding step is essential to reduce particle size and increase the surface area of the organic waste prior to fermentation. As a consequence, solubility and fermentation efficiency are both favoured in the acidogenic fermentation process. Chemical pre-treatment methods using oxidizing agents, alkali, acids and salts are most frequently reported because they require no direct energy input (Mtui, 2010).

5.4. Strategies for improved biohydrogen production

Strategies for improving the biohydrogen production rate and yields have been based on genetic improvement of the microbial strains, or at the fermentative level, on the modelling and optimization of key process parameters, using response surface methodology or artificial intelligence techniques, or on inoculum and substrate pretreatment techniques.

5.4.1. Genetic manipulation of the production strains

Metabolic engineering involves the genetic modification of microorganisms to target and manipulate enzymatic, regulatory, or transport pathways that affect a particular microbial process (Das *et al.,* 2008). Recent success in genome sequencing and gene expression

analysis has enhanced the ability to engineer microorganisms for specific metabolic tasks. Several studies show that H_2 production can be increased by directing the carbon flow toward synthesis of formate. Yoshida *et al.* (2006) have experimentally proved that faster induction of the enzyme formate H_2 lyase (FHL) is possible by elimination of lactate and succinate formation. Increased yields from 1.08 mol/mol glucose to 1.82 mol/mol glucose in the *Escherichia coli* SR15 strain lacking lactate and succinate production have been achieved (Yoshida *et al.,* 2006). Thus, by understanding which metabolic pathways contribute to and regulate the H_2 production, elimination of hydrogen-consuming reactions may be targeted to sustain and regulate the H_2 production rates. Detailed studies can be conducted to use genetic tools to overcome the metabolic barrier by manipulating the electron flux in H_2-producing organisms. The development of microbes that ferment multiple sugars, or which can directly utilize the naturally abundant sugars cellulose/hemicellulose can be targeted (Das *et al.,* 2008).

5.4.2. Applications of response surface methodology for determination of optimum process setpoints

Response surface methodology (RSM) has been widely used in various works to optimize the key parameters for enhanced biohydrogen production. Fermentation optimization based on a statistically planned experiment is a sequential process (Box *et al.,* 1978; Haaland, 1989). First, a large number of continuous factors are screened and insignificant ones are eliminated. The remaining factors could be optimized by response surface modelling. Finally, after model building and optimization, the predicted optimum is verified (Swanson., 1986; Tao *et al.,* 2007). RSM designs are also useful for determining the interaction between the process variables important for the product yield. These include central composite design, mixture design, full factorial design and box–behnken design.

Ghosh *et al.* (2012) used box–behnken design to optimize biohydrogen production, using substrate (glucose), fixed nitrogen, and light intensity during the single-stage photo-fermentation of glucose by the photosynthetic bacterium *Rhodobacter capsulatus*. They realized that the three independent variables, glucose, glutamate, and light intensity, had significant interactive effects on the biohydrogen yield and nitrogenase activity. The model has a coefficient of determination (R^2) of 0.99. The optimized biohydrogen yield shows an 85 % improvement (Ghosh *et al.,* 2012). Xing *et al.* (2011) enhanced biohydrogen production from corn stalk by anaerobic fermentation, using central composite design. The optimum

setpoints of the physico-chemical process parameters were determined. A model with a coefficient of determination (R^2) of 0.96 was generated. Several studies on the optimization of fermentative biohydrogen production by the one-factor-at-a-time method have been reported. This strategy does not depict the interactive effect among the variables and does not guarantee the determination of optimal conditions (Argun *et al.*, 2008; O-Thong *et al.*, 2008). In another study conducted by Liu *et al.* (2011), the optimum conditions for biohydrogen production were predicted using response surface methodology when compost leachate was used as a source of nutrient for fermentative biohydrogen production. The model showed that the maximum cumulative biohydrogen volume (469.74 mL) and molar biohydrogen yield (1.60 mol H_2/mol glucose) could be achieved at 6174.93 mg/L glucose and 3383.20 mg COD/L leachate. A model with a coefficient of determination (R^2) of 0.8281 was generated. These studies have shown that optimization methodologies are crucial for biohydrogen process development.

5.4.3. Artificial neural network in biohydrogen bioprocess development

Artificial neural network (ANN) is a type of artificial intelligence that is inspired by the way the brain processes information. It consists of simple synchronous processing elements called neurons which are connected to each other by links with their own weight factors (Razak *et al.*, 2004). The network needs to learn the connection weights from an available training pattern in order to improve its performance over time. Various aspects have to be considered before a satisfactory neural network model is developed. The development of a neural network model includes database collection, analysis and pre-processing of data, design and training of the neural network, test of the trained network and use of the trained neural network for simulations and predictions (Malinova and Guo, 2004). Jo *et al.* (2011) used ANN for maximizing biohydrogen production in a packed-bed bioreactor. The performance of the bioreactor was also predicted by the model on the key process parameters such as biohydrogen production rate and the metabolites in the effluent. Mu and Yu (2007) used a neural network and genetic algorithm to predict the hydrogen production and the steady-state of an upflow anaerobic sludge blanket (UASB) reactor at various sucrose concentration and Hydraulic Retention Times. Similarly, Guo *et al.* (2008) estimated the biohydrogen yield and the chemical oxygen demand, using ANN in an expanded granular sludge bed (EGSB) reactor. ANNs are useful for prediction of biohydrogen production by their ability to learn complex non-linear input-output relationships, using sequential training procedures, and to adapt themselves to data (Guo *et al.*, 2008; Lo *et al.*, 2008; Nikhil *et al.*, 2008; Rosales-

Colunga *et al.*, 2010).

5.4.4. Lignocellulose substrate pre-treatment strategies

Biological production of hydrogen from glucose is too expensive, and is thus not an economically viable process. Biohydrogen production from renewable sources such as agricultural biomass is economically feasible (Lo *et al.*, 2008). Cellulose is the major constitute of plant biomass and highly available in agricultural wastes and industrial effluents, such as those from the pulp/paper and food industry (Lo *et al.*, 2008; Pataki *et al.*, 2006), and is significant for biohydrogen production. Initial pre-treatment procedures are required to enhance the release of soluble sugars. Mechanical, physical, chemical and biological procedures are often adopted. Mechanical methods involve the breakdown of biomass residues into fine particles, thus increasing the specific surface area for subsequent hydrolysis. Physical treatments such as heating are extensively reported and have been shown to be more effective for disruption of cellulose structure, thereby enhancing the porosity of biomass residues and their accessibility to microorganisms during fermentation. However, this type of pretreatment is energy-consuming and does not remove the lignin content which withstands the enzymatic degradation (Esteghlalian *et al.*, 2000; Zheng *et al.*, 2009).

Most of the chemical pre-treatments that have been assessed to date (typically acid and alkali based methods) have the primary goal of enhancing the accessibility of biohydrogen-producing bacteria to cellulose by solubilizing the hemicellulose and lignin, and to a lesser degree decreasing the degree of polymerization and crystallinity of the cellulosic component and thus allowing biohydrogen-producing bacteria to have access to soluble sugars (Martin and Vermette, 2005). Amongst these pre-treatments technologies, acid pretreatment is considered to be efficient and easy to perform on industrial scale (Pan *et al.*, 2010). Dilute-acid hydrolysis is widely reported as a method for pre-treatment of lignocellulosic materials. Sulphuric acid and hydrogen chloride at concentrations below 4 wt % have been widely used, as they are inexpensive and effective. Dilute acid effectively removes and recovers most of the hemicellulose as dissolved sugars, and glucose yields from cellulose increase with hemicellulose removal to almost 100% for complete hemicellulose hydrolysis (McMillan, 1994).

5.4.5. Inoculum pre-treatment methods

Heat-shock pre-treatment methods have been widely applied to enrich biohydrogen-producing bacteria (Lay, 2001; Wang *et al.*, 2003; Zheng *et al.*, 2009) and eliminate the non-

spore-forming methanogenic bacteria, since hydrogen-producing bacteria, like most *Clostridium sp.*, can form protective spores under extreme conditions. Heat-shock treatment of hydrogen-producing mixed inoculum within a temperature window of 80 °C to 121 °C, and exposure time between 15 min and 120 min are commonly reported. Repeated heat-shock pre-treatments and two-stage cultivation heat-shock pre-treatment (Zheng *et al.,* 2009) have been reported in sucrose medium. Biohydrogen-producing seed has been obtained by treating the sludge by acid at a pH value of 2–4 (Chen *et al.,* 2002; Rosales-Colunga *et al.,* 2010). Zhang *et al.* (2006) applied the method of combined heat-shock and acid-shock on sludge for biohydrogen inoculum pre-treatment. Cai *et al.* (2004) has performed an extensive study on the pre-treatment of sewage sludge by alkaline pre-treatments and found that maximum biohydrogen occurred at initial pH of 11.

5.5. Miniaturized parallel bioreactors in bioprocess development

5.5.1. Miniaturized bioreactors

Bioprocess development for microbial cultivation and optimization are typically performed in expensive, mechanically complex and labour intensive, stirred-tank bioreactors (Zhang *et al.,* 2006). Therefore, microbioreactor technology has been used to address these challenges in order to reduce experimentation costs and speed up the research output. Industries have often employed simplified systems such as microtiter plates, shake flasks, test-tubes and spinner flasks for multi-factorial experimentation which offer ideal strategy to investigate the complex relationships between culture conditions and process outcomes (Bareither and Pollard, 2011; Legmann *et al.,* 2009). Several authors have highlighted the need for miniaturized parallel bioreactors that monitors and controls the physico-chemical parameters for high throughput experimentation (Betts and Baganz, 2006; Box *et al.,* 1978; Hanson *et al.,* 2009; Isett *et al.,* 2007; Martin and Vermette, 2005; Puskeiler *et al.,* 2005; Reis *et al.,* 2006). This is particularly important for multivariate experimentation. A microbioreactor must possess similar characteristics to a bench-scale bioreactor in terms of fermentation conditions, feedback control loops (Gramer and Poeschl, 2000; Hu and Aunins, 1997; Kuwae *et al.,* 2005; Meng *et al.,* 2007), product quality and yield. Recently, some of these reactors have been enhanced with the capability to monitor and control parameters such as optical density (OD), pH, temperature and dissolved oxygen (DO) online and in real time and thereby avoid the need for sample removal (Zhang *et al.,* 2007). The optical sensor technologies have been applied to these bioreactors for online monitoring. Kensy (2010) reported an online monitoring technique in continuously shaken microtiter plates (MTPs) for

detecting the most relevant fermentation parameters such as biomass, fluorescent protein concentrations, pH and dissolved oxygen tension (DOT) in microbial fermentations with *Eschericha coli* and *Hansenula polymorpha* as model organisms. Earlier, Rivera (2004) proposed a parallel microbioreactor with six wells, using optical sensors for monitoring and controlling cell culture conditions. A dissolved oxygen sensor based on the fluorescence quenching of ruthenium diphenylphenanthroline dichloride and an optical sensor based on light transmittance were used in the six-well microbioreactor. These optical sensors were relatively inexpensive to fabricate and well suited for miniaturization and multiplexity.

Maharbiz *et al.* (2004) integrated microtiter plate wells with silicon-monitoring technology in a 250 mL microbioreactor arrays with ion-selective field effect transistor (ISFET) sensors on a commercial printed circuit board. For aeration, oxygen was generated in the bioreactor by hydrolysis of water. The microbioreactor reported by Lamping *et al.* (2003) was a scaled-down version of conventional stirred-tank bioreactors machined in Plexiglas and outfitted with air spargers and a stirring baffle.

Shake flasks are the most common miniature bioreactors and have been estimated to be used in over 90 % of all culture experiments across industry and academia for growing a wide range of microorganisms, e.g. bacteria (Moser *et al.,* 1998), fungi (Tucker and Thomas, 1994), and yeasts (Anderlei and Buchs, 2001) as well as mammalian cells (Girard *et al.,* 2001). They are an inexpensive and effective way of reproducibly performing many types of industrially-relevant cell cultivations for process development (Betts and Baganz, 2006). Shake flask bioreactors have various sizes ranging from milliliters to several litres. These vessels are made of glass or plastic materials, and are operated in a batch or fed-batch mode. The temperature is controlled using incubator or water bath, while the mixing is achieved through linear or orbital shaking. Non-baffled shake flasks can be operated such that bubbles are not formed which provides well defined gas-liquid mass transfer conditions (Zimmermann *et al.*, 2006). Generally, the pH is buffered or not, and poorly controlled.

5.5.2. Parallel bioreactors in bioprocess development

Automated parallel bioreactor systems performing several fermentation processes concomitantly can significantly speed up the development of biohydrogen production processes as well as other bioprocesses (Bao *et al.,* 2004; Rocha *et al.,* 2006). The high throughput of these systems leads to reduction in time, labour intensity, media cost, and space requirements, as compared to conventional bioreactors (Bao *et al.,* 2004; Betts and Baganz,

2006). Different strategies have been proposed for parallel bioprocess development and optimization. Jo *et al.* (2008) described the use of up to 48 stirred-tank parallel bioreactors for biohydrogen production. This approach involved gas-inducing stirrers for stirred-tank bioreactors on a 10 mL scale. To ensure an easy parallelization, a magnetic inductive drive was developed which allowed for the parallel operation of the 48 stirred-tank bioreactor in a bioreaction block. In this type of bioreactor, parameters such as pH, temperature and dissolved oxygen were monitored and controlled online. The Sixfors benchtop device (Infors AG, Bottmingen, Switzerland) has six fermenters operating in parallel (Betts and Baganz, 2006) and the Cellstation bioreactors (Fluorometrix Corporation, Stow, MA) allow 12 miniature stirred-tank bioreactors to be operated in parallel. Parameters such as pH and temperature are controlled online in these bioreactor systems, whereas agitation is achieved through baffles and impellers (Zhang *et al.*, 2007). The miniaturization and parallelization of bioreactors for biohydrogen is an attractive approach for development of this process.

5.6. Application of miniaturized parallel bioreactors in biohydrogen research

Multivariate fermentative biohydrogen research has been carried out in miniaturized parallel bioreactors. A working volume of 5 mL to 500 mL and bioreactor parallelization ranges from 3 to 50 have been used (Table 5.1). Various substrate types such as food, dairy, and agricultural wastes are used. A parallelization up to 50 bioreactors has been used with cellulose as a substrate and up to 32 bioreactors on food wastes. Although the parallelization level is correlated to the number of parameters under investigation rather than the nature of the substrate, the miniaturization scale is limited with the complexity of the medium, and glucose substrate for biohydrogen research has been used in a miniaturized bioreactor of a 5 mL working volume.

The monitoring and control strategies for the physico-chemical parameters in these bioreactors for biohydrogen research are presented in Table 5.2. The pH value is either monitored with miniaturized pH probes and controlled by addition of acid or base, or not controlled and the initial pH value of the culture is adjusted at a desired setpoint, despite the fast drift in pH setpoints during the fermentation process as result of substrate types or metabolic activities. Temperature is regulated by water baths, incubators or shakers whereas agitation is achieved by magnetic stirrers or shaking water baths. In some cases, mixing is done manually at regular time intervals; however this method lacks consistency, reproducibility, efficiency and reliability.

In these reactors, the hydrogen fraction of the generated biogas is measured using real-time hydrogen sensors or gas chromatography which has the ability to measure other compounds produced during the fermentation process, but it has the traditional drawbacks of the class of problems associated with offline samplings. The cumulative hydrogen gas volume determined by gas chromatography is calculated according to Equation 1.

$$V_{H,i} = V_{H,i-1} + C_{H,i}\,(V_{G,i} - V_{G,i-1}) + V_H\,(C_{H,i} - C_{H,i-1}) \quad (1)$$

$V_{H,i}$ and $V_{H,i-1}$ are the cumulative hydrogen gas volume at the current (i) and the previous (i-1) time intervals, $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volumes in the current and previous time intervals, $C_{H,i}$ and $C_{H,i-1}$ are the fractions of hydrogen gas in the headspace of the bottle measured using gas chromatography in the current and previous intervals, and V_H is the total volume of headspace in the bottle (Chong *et al.*, 2009).

Table 5.1: Miniaturized parallel bioreactors used for fermentative biohydrogen production.

Bioreactor size (ml)	Working volume (ml)	Bioreactor parallelization	Substrate types	Reference
300–500	400	3 to 4	Peptone	Zabut *et al.* (2006)
	450	16	Glucose and compost	Liu *et al.* (2011)
	500	-	Glucose	Nath *et al.* (2005)
250–300	250	-	Wheat starch	Oztekin *et al.* (56)
	285	-	Ground wheat	Argun *et al.* (2008)
200–250	200	32	Food waste	Kim *et al.* (2004)
	200	16	Glucose	Venkata Mohan *et al.* (2007)
	200	8	Dairy wastewater	Venkata Mohan *et al.* (2007)
	200	7	Rice slurry	Fang *et al.* (2006)
150–180	150	-	Sucrose	Van Ginkel *et al.* (2001)
	150	-	Wheat straw waste	Fang *et al.* (2006)
	180	12	Market waste	Venkata Mohan *et al.* (2009)
100–150	100	3	Apple pomace	Wang *et al.* (2010)
	100	-	Glucose	Guo *et al.* (2009)
	120	18	OFMSW	Lay *et al.* (1999)
	125	27	Various metals	Lin and Lay (2005)
50–100	60	4	Rice straw	Chen *et al.* (2012)
	50	-	Corncob	Pan *et al.* (2010)
	50	-	Glucose	Oh *et al.* (2003)
	50	-	Filtrate of biosolids	Wang *et al.* (2003)
	50	12	Stale corn	Wang *et al.* (2012)
	60	10	Mushroom waste	Lay *et al.* (2012)
	65	48	POME	O-Thong *et al.* (2008)
	80	16, 4 and 50	Cellulose	Lay (2001)
	40	-	Sucrose	Zhu and Béland (2006)
	80	11	Lactose, cheese whey	Davilla-Vazquez *et al.* (2008)
20–50	20	5	Xylose	Kongjan *et al.* (2009)
	30	10	Glucose	Singh and Gu (2010)
	30	5	Cassava and food waste	Zong *et al.* (2009)
	34	5	Cassava and food waste	Zong *et al.* (2009)
	39	6	Sucrose	Tao *et al.* (2007)
<20	5	6 and 10	Zinc, iron and glucose	Ooshima *et al.* (1998)

−: Not stated.

Table 5.2: Monitoring and control of process variables in miniaturized parallel bioreactors for biohydrogen research.

Bioreactor size (mL)	Control methods			H_2 sensing procedure	Reference
	pH	Temperature	Agitation		
300–500	pH probe	Water bath	Magnetic stirrer bar	GC (Hewlett Packard 5890, series II)	Zabut *et al.* (2006)
	Initial pH adjusted	Water bath	Orbital shaker	GC (Perkin-Elmer,USA)	Nath *et al.* (2005)
250–300	Initial pH adjusted	Incubator	Mixed manually	GC (Agilent, 6890)	Argun *et al.* (2008)
200–250	pH probe	Temp regulated shaker	Orbital shaking	GC (Gow Mac, series 580)	Kim *et al.* (2004)
	Initial pH adjusted	Temp regulated shaker	Orbital shaking	Microprocessor H_2 sensor (ATMI GmBH inc., Germany)	Venkata Mohan *et al.* (2007)
	pH sensor and controller	Temp regulated shaker	Orbital shaking	GC (Hewlett-Packard 589011, USA)	Fang *et al.* (2006)
150–180	Initial pH adjusted	Incubator	Orbital shaking	GC (Gow Mac, series 580)	Van Ginkel *et al.* (2001)
	Initial pH adjusted	Temp regulated shaker	Orbital shaking	Microprocessor H_2 sensor (ATMI GmBH inc., Germany)	Venkata Mohan *et al.* (2009)
100–150	Initial pH adjusted	Not stated	Magnetic stirring	GC (SP 6890)	Wang *et al.* (2010)
	Initial pH adjusted	RCC	Rotatory shaking	GC (Shimadzu 8A)	Lay *et al.* (1999)
	Initial pH adjusted	Incubator	Orbital shaking	GC (Model 310, SRI Instruments, Torrance, CA)	Baghchehsaraee *et al.* (2008)
50–100	Initial pH adjusted	Incubator	Orbital shaking	GC (Agilent, 4890D)	Pan *et al.* (2010)
	Initial pH adjusted	Incubator	No shaking	GC (Agilent, 4890D)	Wang *et al.* (2012)
	Initial pH adjusted	Temperature regulated orbital shaker	Orbital shaker	GC(8700T, China)	Lay *et al.* (2012)
	Initial pH adjusted	RCC	Rotatory shaking	GC (Shamadzu 8A)	Lay (2001)
	Initial pH adjusted	Incubation room	Horizontal shaking	GC (Agilent 68090N, Germany)	Davila-Vazquez *et al.* (2008)
20–50	Initial pH adjusted	Incubator	Orbital shaking	GC (Microlab, Arhus, Denmark)	Kongjan *et al.* (2009)
	Initial pH adjusted	Incubator	Not stated	GC (Techcomp, 7900, China)	Zong *et al.* (2009)
<20	Initial pH adjusted	Incubator	Not stated	GC (Producer not stated)	Ooshima *et al.* (1998)

H_2: Hydrogen gas; RCCS: Rotatory Cell Culture System, OFSMW: Organic Fraction of Solid Municipal Waste, GC: Gas Chromatography.

5.7. Proposed features for novel miniaturized parallel biohydrogen bioreactors

Biohydrogen process development will inherently gain from bioreactor miniaturization and parallelization at least to understand the synergistic or antagonistic effects of multiple parameters' interaction on hydrogen yield and production rate. These reactors will need additional considerations on (i) parallelization, (ii) maintenance of the pH control setpoint, and (iii) real-time measuring of hydrogen fraction.

5.7.1. Parallelization:

Production of biohydrogen is more economically feasible on multiple-waste substrate streams, incorporating several interacting key elements which are furthermore influenced by process physico-chemical parameters. Multifactorial experimentation is thus required for process model development on these inputs.

5.7.2. Maintenance of the pH control setpoint:

In most reported microbioreactors, the initial pH value of the culture is adjusted, with no further attempt to control. This variable does not remain constant, but drifts during the process, influencing metabolic fluxes, thus altering the yield and productivity data.

5.7.3. Real-time measuring of hydrogen fraction:

To date, the offline gas chromatography analysis of the evolving fraction has been the prime procedure. Its shortcoming is an overestimation of the cumulative volume of the hydrogen biogas fraction, as the sampling interval increases. In our laboratory, cumulative biohydrogen volume of 135.60 mL and 157.61 mL was found while comparing two sampling intervals of 1 min and 12 h, respectively, on the same process.

5.8. Conclusions

A critical challenge for hydrogen fermentation is the low hydrogen conversion efficiency (Das *et al.*, 2008). This may be overcome by using industrial, municipal or agricultural wastes which are abundant, costless and renewable. However, multivariate experimentations will be required to generate accurate fermentation information which is translatable into actionable intelligence for biohydrogen process scale-up. This requires novel bioreactor configurations with a high level of parallelization coupled with integration of on-line monitoring techniques for detecting the most relevant fermentation parameters in biohydrogen production. The development of micro-sensors is necessary in order to provide real-time and reliable bioprocess information and also to determine suitable parameter

setpoints for maximum biohydrogen production.

5.9. References

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CHAPTER 6

Conclusions and Recommendations for future work

6.1. Conclusions

In this study, the potential of using Organic Fraction of Solid Municipal Waste (OFSMW) for biohydrogen process modelling and optimization was demonstrated. Based on these results, the following conclusions can be inferred:

6.1.1. A maximum hydrogen yield of 57.73 ml H_2/g TVS is achievable when OFSMW is used a sole substrate at optimum setpoint conditions of 40.45 g/l, 7.9, 30.29 °C and 86.28 h for substrate concentration, pH, temperature and Hydraulic Retention Time (HRT) respectively. These results demonstrate that a suitable optimization of the key physico-chemical parameters is a critical step for biohydrogen process development.

6.1.2. The feasibility of a large-scale stable biohydrogen production on OFSMW was demonstrated. A hydrogen fraction of 46.7% and hydrogen yield of 246.93 ml H_2/g TVS were obtained from the semi-pilot scale process.

6.1.3. This study showed the possibility of generating electricity using the process effluents of biohydrogen production coupled to a Microbial Fuel cell. A maximum power density of 0.21 W/m^2 and COD removal of 50.1% were obtained from Microbial Fuel Cell (MFC) process. The combination of dark fermentation process and MFCs may significantly enhance the overall hydrogen-production rates and yields. Moreover, integration of these two-stage processes may eliminate high-cost and energy-intensive detoxification processes used for treatment of wastewater and biomass effluents.

6.2. Recommendations for future work

In order to realize the potential of industrial-scale biohydrogen production process, several recommendations are proposed for future studies:

6.2.1. Employing organic waste materials for biological hydrogen production processes will significantly improve its process economics by reducing the production cost, since they are abundant, costless, renewable, and have high hydrogen efficiency.

6.2.2. The molecular study of hydrogen-producing microorganisms will generate more knowledge on the metabolic pathways of hydrogen production. By understanding which metabolic pathways contribute to and regulate the hydrogen production, elimination of hydrogen-consuming reactions may be targeted to sustain and regulate the hydrogen production rates. Studies can also focus on genetic tools to overcome the metabolic barriers by manipulating the electron flux in hydrogen-producing organisms.

6.2.3. Integration of two-stage processes has been shown to be effective for maximum hydrogen conversion efficiency. These include hydrogen and methane generation, hydrogen and microbial fuel cells, hydrogen and microbial electrolysis cell. However the cost analysis for these processes will need to be considered at scale-up level.

6.2.4. Multifactorial experimentations will also be required to generate reliable fermentation data which is translatable into actionable intelligence for biohydrogen process scale-up. This requires novel bioreactor configurations with a high level of parallelization coupled with integration of on-line monitoring techniques for detecting the most relevant fermentation parameters in biohydrogen production. The development of micro-sensors is necessary in order to provide real-time and reliable bioprocess information and also to determine suitable parameter setpoints for maximum biohydrogen production.

APPENDIX

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A two-stage modelling and optimization of biohydrogen production from a mixture of agro-municipal waste

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ABSTRACT

A two-stage modelling and optimization of biohydrogen production is reported. A mixture design was used to determine the optimum proportions of bean husk (BH), corn stalk (CS), and organic fraction of solid municipal waste (OFSMW). The optimum operational set-points for substrate concentration, pH, temperature and hydraulic retention time (HRT) were further investigated using the box-behnken design. The quadratic polynomial model from the mixture design had a coefficient of determination (R^2) of 0.9427 and the optimized mixtures were in the ratio of OFSMW:BH:CS = 30:0:0 and OFSMW:BH:CS = 15:15:0 with yields of 56.47 ml H_2/g TVS and 41.16 ml H_2/g TVS respectively. Optimization on physico-chemical process parameters on the improved substrate gave the setpoints of 40.45 g/l, 7.9, 30.29 °C, 86.28 h for substrate concentration, pH, temperature and HRT respectively having a predicted H_2 yield of 57.73 ml H_2/g TVS. Model validation gave 58.62 ml H_2/g TVS, thus an improvement of 3.8% on the optimized mixture. Biohydrogen production can be significantly enhanced by a suitable mixture of agro-municipal waste and operational optimal setpoints.

1. Introduction

The dependence on fossil fuels poses great challenges to both the climatic and environmental systems, thus prompts an urgent need for the development of non-polluting and renewable energy sources. Biohydrogen is an excellent alternative energy since its combustion produces only water. It has a high energy yield (122 kJ/g) which is 2.75 times greater than its equivalent of hydrocarbon fuels [1,2]. Its production via the fermentative route is more environmentally friendly, less energy intensive compared to the chemical hydrogen production methods [3]. Despite its many benefits, progress towards a biohydrogen economy has been hindered by a low yield on costly substrates.

Agricultural and organic municipal waste substrates are abundant, costless, renewable and can potentially be used as substrates for bioenergy production. An estimated annual yield of 118 × 10^9 tons of dry biomass is generated worldwide [4], the energy equivalent of 60–70 billion tons of crude oil. South Africa generated 59 million tons of general waste in 2011. The agricultural and municipal fractions were estimated at 2.95 and 7.88 million tons respectively, and only 35% of these, mainly of municipal types, were recycled [5]. The rest were burnt or disposed of in landfills. Biohydrogen production

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using these substrates will not only alleviate environmental hazards but also save the energy demands needed to treat them. This work investigates the optimum proportion of bean husk (BH), corn stalk (CS) and organic fraction of solid municipal waste (OFSMW) for biohydrogen production using a mixture design. Furthermore, the effects of input parameters of substrate concentration, pH, temperature and hydraulic retention time (HRT) on hydrogen response, using the mixed substrate, are modelled and optimized.

2. Materials and methods

2.1. Determination of optimum substrate composition using a mixture design

2.1.1. Mixture design and substrate pre-treatment

A mixture design was used to determine the optimum proportion of co-substrates of BH, CS and OFSMW for biohydrogen production. Fourteen different mixtures were generated with varying proportions of these substrates to a total concentration of 30 g/l (Table 1). The agricultural waste of BH and CS were collected from the Ukulinga Research Farm, University of KwaZulu-Natal, Pietermaritzburg, South Africa. They were dried at room temperature, reduced in particle size to 2.00–2.80 mm, and kept for further use. OFSMW was simulated according to Gomez et al. [6], and was made up of 10% apple, 10% orange, 35% cabbage, 35% potato, 8% bread, and 2% paper. The total volatile solids (TVS) content of experimental mixed crop residues was determined according to Equation (1).

$$\mathrm{TVS} = \frac{\text{Weight of dried waste} - \text{Weight of ash}}{\text{Weight of dried waste}} \times 100\% \quad (1)$$

2.1.2. Inoculum development

Hydrogen-producing mixed consortia used in the study was obtained from the anaerobic sludge collected from the Darvill waste water treatment plant, Pietermaritzburg, South Africa.

Table 1 – Biohydrogen production from mixture design.

Batch	A: OFSMW (g/l)	B: bean husk (g/l)	C: corn stalk (g/l)	H_2 yield (ml/g TVS)
1	30	0	0	56.47
2	5	5	20	11.57
3	0	30	0	17.67
4	0	15	15	12.73
5	20	5	5	40.54
6	15	15	0	33.4
7	15	15	0	23.75
8	30	0	0	54.22
9	0	0	30	3.9
10	10	10	10	16.37
11	15	0	15	24.05
12	0	0	30	3.68
13	5	20	5	14.56
14	0	30	0	31.04

Previous studies with this inoculum showed the presence of endospore forming clostridia (unpublished results). The sludge was heated at 100 °C for 30 min to deactivate the hydrogen consuming methanogenic bacteria, thus enabling the survival of hydrogen producing endospore forming bacteria.

2.1.3. Fermentation process

The fermentation processes were carried out in parallel bioreactors of 250 ml modified Erlenmeyer flasks. Reactors were fed with co-substrates at concentrations as stated in the mixture design to a total value of 30 g/L, supplemented with inorganic salts (all in g/L): NH_4Cl 0.5, KH_2PO_4 0.25, K_2HPO_4 0.25, $MgCl_2.6H_2O$ 0.3, $FeCl_3$ 0.025, $ZnCl_2$ 0.0115, $CuCl_2$ 0.0105, $CaCl_2$ 0.005 and $MnCl_2$ 0.015. They were inoculated with 10 ml of pre-treated sludge and made up to a working volume of 100 ml with distilled water. Anaerobiosis was created by flushing the reactors with nitrogen gas for 1 min. The initial pH was adjusted to 6.5. Fermentations were carried out in duplicate in a water bath shaker with operational setpoints of 60 rpm, 35 °C and 72 h for agitation, temperature and HRT respectively.

2.1.4. Analytical procedure

The evolving biogas volume was measured using the water displacement method [7]. This method is reliable and offers the possibility of being interfaced with a computer module. The hydrogen fraction of mixed biogas was determined using the hydrogen sensor BCP-H_2 (Bluesens, Germany) with a range of 0–100% and a measuring principle based on thermal conductivity detector. The cumulative volume of biohydrogen produced was computed regularly according to Equation (2).

$$V_{H,i} = V_{H,i-1} + C_{H,i}\left(V_{G,i} - V_{G,i-1}\right) + V_H\left(C_{H,i} - C_{H,i-1}\right) \quad (2)$$

$V_{H,i}$ and $V_{H,i-1}$ are cumulative hydrogen gas volumes at the current (i) and previous (i−1) time intervals, $V_{G,i}$ and $V_{G,i-1}$ the total biogas volumes in the current and previous time intervals, $C_{H,i}$ and $C_{H,i-1}$ the fraction of hydrogen gas in the headspace of the reactor in the current and previous time intervals, and V_H the total volume of headspace in the reactor [8].

2.1.5. Modelling and optimization of mixtures

The experimental data were used in multiple regression analysis to develop a quadratic model that relates hydrogen production to the proportions of BH, CS and OFSMW in the mixture, according to Equation (3).

$$Y = \alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_3 + \alpha_{11} x_1^2 + \alpha_{22} x_2^2 + \alpha_{33} x_3^2 + \alpha_{12} x_1 x_2 + \alpha_{13} x_1 x_3 + \alpha_{23} x_2 x_3 \quad (3)$$

where Y is the hydrogen response, α_0 is the intercept, $\alpha_1 x_1$ to $\alpha_3 x_3$ represents the linear blending portion, $\alpha_{11} x_1^2$ to $\alpha_{33} x_3^2$ are quadratic coefficients and $\alpha_{12} x_1 x_2$ to $\alpha_{23} x_2 x_3$ are the interaction coefficients.

The significance of the model was assessed by the Analysis of Variance (ANOVA) using Design Expert software (Stat Ease, Inc, USA). The optimum proportion of the co-substrates in the mixture was obtained by solving the quadratic equation. The optimum substrate concentration and other physico-

chemical process variables were subsequently investigated using the box-behnken design.

2.2. Determination of optimum parameter setpoints using the box-behnken design

2.2.1. Experimental setup

The box-behnken design was used to model the relationship between the physico-chemical variables of substrate concentration, pH, temperature and HRT on hydrogen response, and to determine the optimum operational setpoints. Twenty-nine fermentation batches with varied combinations of input parameters were generated (Table 4) for experimentation. Parallel bioreactors, made up of modified Erlenmeyer flasks, were fed with the previously optimized medium, inoculated with 10 ml of pre-treated sludge and made up to 100 ml with distilled water. Fermentation processes were carried out as described in the previous stage, but with the physico-chemical parameters varied according to the box-behnken design.

2.2.2. Modelling and optimization of physico-chemical variables

The experimental data obtained from this stage were used in multiple regression analysis to develop a quadratic model that relates hydrogen production to the considered physico-chemical parameters. This model was subjected to the ANOVA. The optimum operational conditions for H_2 production were obtained by solving the quadratic equation.

3. Results and discussion

3.1. Process model on co-substrate inputs

Experimental data from the mixture design (Table 1) were used to fit a quadratic model relating the OFSMW, BH and CS to hydrogen production. Analysis of variance of the model (Table 2) gave a coefficient of determination of 0.94; thus 94% of the variation in observed data can be explained by the model. The significance of the model was confirmed by the F and P values of 26.32 and 0.0001 respectively. The model can be mathematically expressed according to Equation (4).

$$Y = +44.32A + 18.62B + 3.07C - 36.57AB - 15.34AC - 10.20BC \quad (4)$$

where Y represents H_2 production in ml H_2/g TVS. The coefficient of estimates are shown in Table 3, where A, B and C are the linear coefficients of OFSMW, BH and CS respectively and AB, AC and BC are the interactive coefficient of OFSMW and BH, OFSMW and CS, and BH and CS respectively.

Table 2 – Analysis of variance of the mixture model.

Source	Sum of squares	df	Mean of squares	F-value	P-value	R-square
Model	2132.92	54	26.58	26.32	0.0001	0.9427

df: degrees of freedom, F-value: Fisher–Snedecor distribution value, P-value: Probability value.

Table 3 – Coefficients of estimates of the mixture model and their confidence intervals.

Component	Coefficient estimate	df	Standard error	95% CI Low	95% CI High	VIF
A	44.32	1.00	2.79	37.88	50.77	1.62
B	18.62	1.00	2.79	12.18	25.07	1.62
C	3.07	1.00	2.79	−3.37	9.50	1.50
AB	36.57	13.22	1.00	−67.06	−6.09	1.76
AC	15.34	15.84	1.00	−51.88	21.19	1.55
BC	10.20	15.84	1.00	−46.74	26.34	1.55

df: degrees of freedom, 95% CI Low: 95% Confidence Intervals (Low limit), 95% CI High: 95% Confidence Intervals (High limit), VIF: Variance Inflation Factor.

3.2. Interaction of co-substrates on biohydrogen output and optimization

The hydrogen production from various mixtures, under similar fermentation conditions ranged from 3.68 to 56.47 ml H_2/g TVS (Table 1). This emphasizes the sensitivity of biohydrogen fermentation on substrate composition, as observed earlier by Zhang et al. [9]. Hydrogen yields of 56.47, 31.04 and 3.9 ml H_2/g TVS were obtained when OFSMW, BH and CS were used as the sole substrate respectively, and a consistently high hydrogen production was observed in various mixtures containing the OFSMW (batch 5, 6, 7 and 11). A plausible contribution to a high hydrogen production on OFSMW may be its relative higher nutritional composition. A similar high hydrogen production pattern on OFSMW was observed by Dong et al. [10], and was attributed to its rich contents of carbohydrates, lipids and proteins required for hydrogen production. A 14 times decrease in H_2 production was obtained when comparing CS to OFSMW as the sole substrate for fermentative H_2 production. This relatively low yield on CS may be linked to the complexity of the polymer structure requiring an acidic or thermal pretreatment, which at industrial scale might substantially impact on process economics. With an HCl pretreatment of CS at 90 °C for 2 h, Wung et al. [11] achieved a hydrogen yield of 126.22 ml/g CS. These observations might suggest that a pretreated CS releases higher amounts of soluble sugars into the medium than OFSMW, but, however, the pattern and the cost/benefit analysis will need to be investigated.

The interactive effect of the mixture on hydrogen response is illustrated on a triangular response surface graph and the contour map plot (Fig. 1a–b). It is observed that hydrogen production was maximum in a mixture having the highest concentration of OFSMW and progressively decreased along the axes OFSMW-BH and OFSMW-CS. A very low hydrogen response was obtained when BH and CS alone were used in the mixture, in any proportion. The optimum proportion of OFSMW, BH and CS for hydrogen production was determined by solving the quadratic model equation using the numerical method of Myers and Montgomery [12]. Two solutions were selected: a mixture of 15 g/l OFSMW, 15 g/l BH and 0 g/l CS predicting a cumulative H_2 production of 41.16 ml H_2/g TVS,

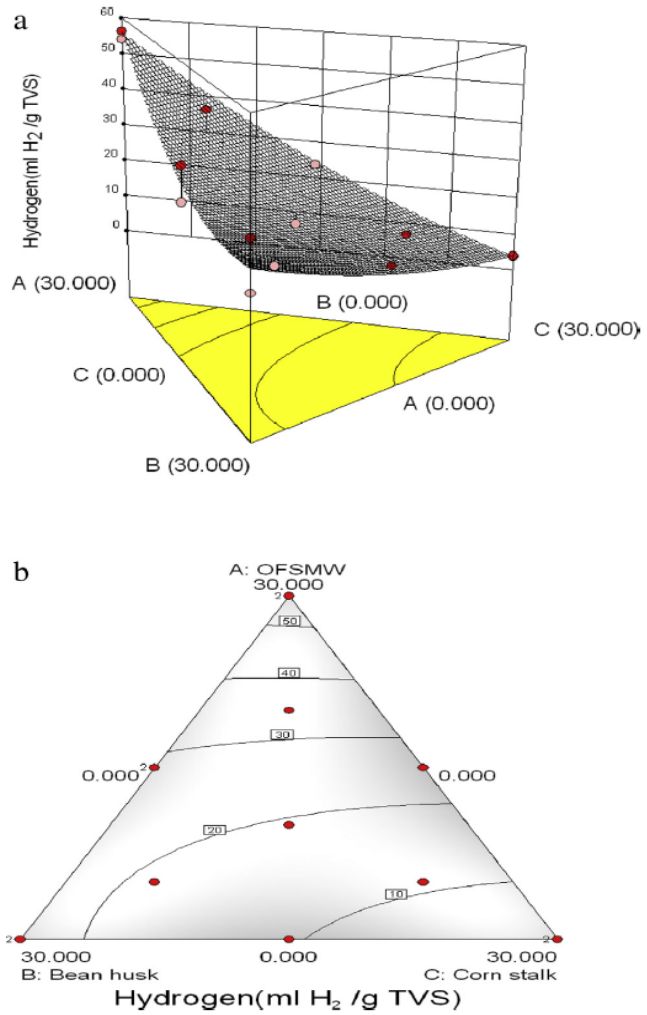

Fig. 1 – a Hydrogen response surface graph from mixture. b Hydrogen response contour plot from mixture.

and a mixture of 30 g/l OFSMW, 0 g/l BH and 0 g/l CS with a cumulative H_2 production of 56.47 ml H_2/g TVS. It is expected that a viable production of biohydrogen on a large scale will depend on the distribution and availability of waste substrate types. Hence, under certain conditions, a mixture of OFSMW and BH may be used instead of OFSMW as a unique substrate. However, in this study further optimization was based on OFSMW as the sole substrate as derived from the optimized mixture design.

3.3. *Process model based on physico-chemical input parameters*

Experimental data obtained from the box-behnken design (Table 4) were used to develop a second order polynomial equation whose coefficients were determined by multiple regression analysis. The suitability of the model was assessed using the ANOVA (Table 5). The high model F value (3.77) and low P value (0.0092) imply that the model is significant. A

Table 4 – Biohydrogen production from the box-behnken design.

Batch	Substrate conc. (g/l)	HRT	pH	Temperature °C	H_2 yield (ml H_2/g TVS)
1	50	53	3	34.5	14.95
2	32.5	53	8	39	7.09
3	50	53	5.5	30	5.95
4	32.5	53	5.5	34.5	41.44
5	32.5	10	3	34.5	0.077
6	32.5	53	8	30	57.65
7	15	96	5.5	34.5	10.11
8	32.5	53	5.5	34.5	15.64
9	32.5	53	5.5	34.5	10.2
10	50	10	5.5	34.5	0.431
11	32.5	53	5.5	34.5	30.55
12	50	53	5.5	39	2.89
13	32.5	53	3	30	0.526
14	32.5	53	3	39	0.676
15	15	53	3	34.5	14.30
16	15	53	8	34.5	11.41
17	32.5	10	5.5	39	0.545
18	32.5	96	5.5	39	0.264
19	32.5	96	5.5	30	14.66
20	15	53	5.5	39	0.222
21	32.5	96	3	34.5	0.158
22	50	53	8	34.5	48.08
23	15	10	5.5	34.5	0.258
24	50	96	5.5	34.5	1.13
25	15	53	5.5	30	1.2
26	32.5	10	5.5	30	0.583
27	32.5	53	5.5	34.5	32.38
28	32.5	96	8	34.5	46.18
29	32.5	10	8	34.5	0.973

coefficient of determination R^2 of 0.7903 was obtained, thus 79.03% of the variability observed in the data can be accounted for by the model. The model's coefficient of estimates are shown in Table 6, where A, B, C, D are the linear coefficients for substrate concentration, HRT, pH and temperature. The magnitude of the coefficient has a direct contribution to the model output. Hence, C, BC, AC and B with coefficient values of 11.72, 11.28, 9.0 and 5.80 have a greater impact on hydrogen response compared to the remaining linear and interactive input effects. This model was expressed mathematically according to Equation (5).

$$Y = +26.04 + 2.99A + 5.80B + 11.72C - 5.74D - 2.29AB + 9.00AC - 0.52AD + 11.28BC - 3.59BD - 12.68CD - 9.17A^2 - 13.61B^2 + 2.23C^2 - 11.50D^2 \quad (5)$$

where Y is the hydrogen yield in ml H_2/g TVS; A, B, C and D are linear coefficients, AB to CD are the interactive coefficients of

Table 5 – ANOVA of the box-behnken derived model.

Source	Sum of squares	df	Mean of squares	F-value	P-value	R-square
Model	6366.59	14	454.76	3.77	0.0092	0.7903

df: degrees of freedom, F-value: Fisher–Snedecor distribution value, P-value: Probability value.

Table 6 – Coefficients of estimates for the box-behnken model and their confidence intervals.

Factor	Coefficient estimate	df	Standard error	95% CI Low	95% CI High	VIF
Intercept	26.04	1	4.91	15.50	36.58	
A	2.99	1	3.17	−3.81	9.80	1.00
B	5.80	1	3.17	−1.00	12.60	1.00
C	11.72	1	3.17	4.92	18.52	1.00
D	−5.74	1	3.17	−12.54	1.06	1.00
AB	−2.29	5.49	1	−14.07	9.49	1.00
AC	9.00	5.49	1	−2.78	20.78	1.00
AD	−0.52	5.49	1	−12.30	11.26	1.00
BC	11.28	5.49	1	−0.50	23.06	1.00
BD	−3.59	5.49	1	−15.37	8.19	1.00
CD	−12.68	5.49	1	−24.46	−0.90	1.00
A^2	−9.17	4.31	1	−18.42	0.083	1.08
B^2	−13.61	4.31	1	−22.86	−4.36	1.08
C^2	2.23	4.31	1	−7.02	11.48	1.08
D^2	−11.50	4.31	1	−20.75	−2.25	1.08

df: degrees of freedom, 95% CI Low: 95% Confidence Intervals (Low limit), 95% CI High: 95% Confidence Intervals (High limit), VIF: Variance Inflation Factor.

parameters on hydrogen production and A^2 to D^2 are the quadratic coefficients.

3.4. *Interaction of physico-chemical parameters on hydrogen production*

Biohydrogen yield under different physico-chemical parameters varied from 0.077 to 57.65 ml H_2/g TVS (Table 4). Analysis of the linear effect of parameters on hydrogen yield pattern indicated that at low setpoint values of HRT, pH, temperature and substrate concentration, low yields of hydrogen were obtained (batch 5, 17 and 23). The interaction of various physico-chemical parameters on hydrogen response taken pairwise with other parameter setpoints maintained at their median values are shown on three dimensional response surface graphs (Figs. 2–6).

In Fig. 2, the interactive effects of HRT and substrate concentration on hydrogen response has a concave shape

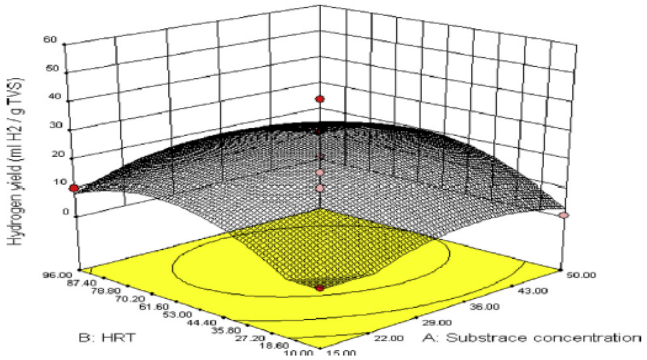

Fig. 2 – Hydrogen response surface graph exhibiting the interactive effects between HRT (hours) and substrate concentration (g/l). Other variables were held at their median values.

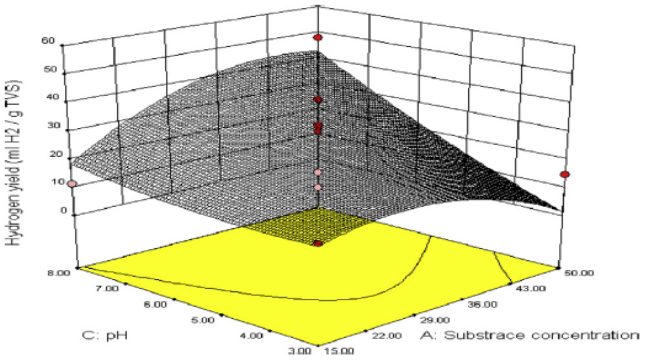

Fig. 3 – Hydrogen response surface graph exhibiting the interactive effects between pH and substrate concentration (g/l). Other variables were held at their median values.

indicating that the optimum setpoints were within the search range, and a peak production above 20 ml H_2/g TVS was observed within the ranges of 48–87 h and 20–42 g/l of HRT and substrate concentration respectively. Fan et al. [13] reported a remarkable increase in H_2 yield with the increase in substrate concentration in the range of 5–20 g/l. But it is believed that at a very high substrate concentration, the accumulation of volatile fatty acids increases. In addition, hydrogen high pressure inhibits the hydrogenase activity [13].

The synergistic effect of pH and substrate concentration (Fig. 3) showed that at pH values between 7 and 8, an increase of OFSMW concentration from 20 to 42 g/l resulted in more hydrogen production. Conflicting optimum pH setpoint values ranging from 6 to 9 have been reported for fermentative biohydrogen production. This might be attributed to the experimental setup, as very often only the initial pH value is reported without further control feedback or buffer system to stabilise the setpoint, despite the fact that fermentation processes are known to exhibit a highly nonlinear pH behaviour as a function of inoculum source and substrate type. More so, even when pH control additives are intermittently used in shake flasks, it is not known how fast these liquid additions are mixed with the broth due to the poor mass transfer in these systems, and it has been demonstrated that

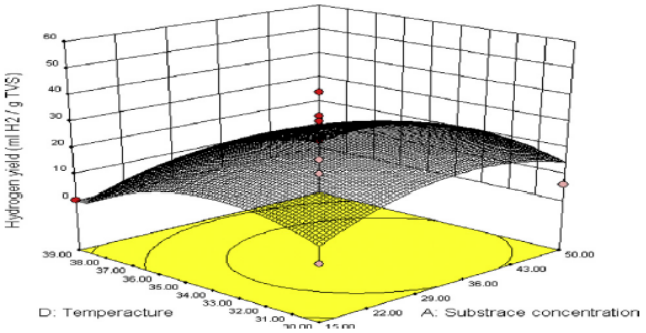

Fig. 4 – Hydrogen response surface graph exhibiting the interactive effects between temperature (°C) and substrate concentration (g/l). Other variables were held at their median values.

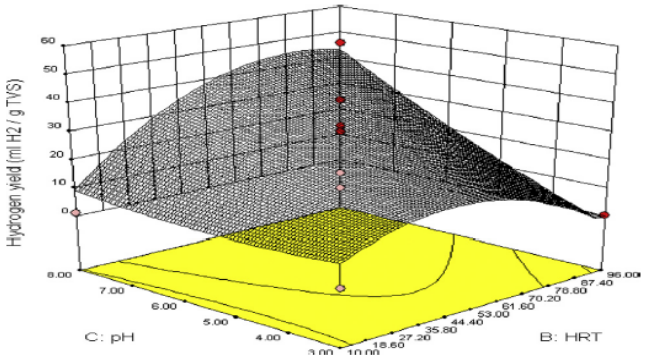

Fig. 5 – Hydrogen response surface graph exhibiting the interactive effects between pH and HRT (hours). Other variables were held at their median values.

microorganisms can swiftly change their metabolic fluxes within a time scale of less than a second [14–16].

Considering the process temperature and substrate concentration, it was observed that at temperatures above 30 °C, a further increase of substrate feed from 15 g/l resulted in an increase in biohydrogen production (Fig. 4). Temperature affects the maximum specific growth, substrate utilization rate and the metabolic pathway of microorganisms, resulting in a shift of by-product compositions [17–19]. Several studies have reported that thermophilic fermentations are favourable for H_2 production compared to mesophilic fermentations. This may be attributed to the fact that these conditions lower the growth rate of hydrogen consuming bacteria [20–22]. Hydrogen yield and production rates of thermophilic bacteria, growing at temperature above 60 °C, often show higher values as compared to those of mesophilic bacteria growing at moderate temperatures [23,24]. Nevertheless, there are specific constraints for H_2 production by thermophiles and extreme thermophiles, one of them being associated with low bacterial cell density, which results in rather moderate H_2 productivity. As illustrated in Fig. 5, at a pH value of about 7, an increase in HRT within the window of 55–87 h resulted in more hydrogen yield. At pH values below 4, very low hydrogen is produced. Peak hydrogen production was obtained within a window of 60–87 h, 30–35 °C for HRT and temperature respectively (Fig. 6).

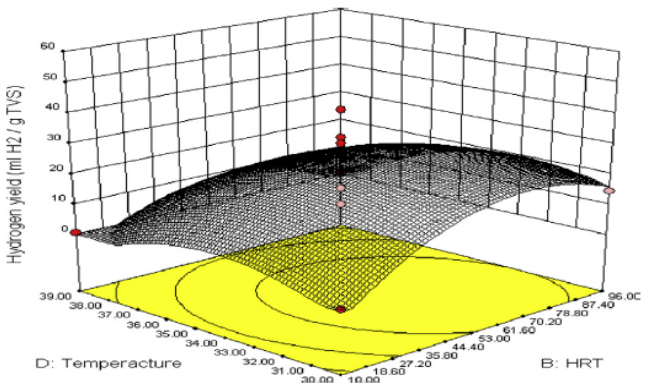

Fig. 6 – Hydrogen response surface graph exhibiting the interactive effects between temperature (°C) and HRT (hours). Other variables were held at their median values.

3.5. *Optimization of biohydrogen production using the box-behnken design*

The optimum operational setpoints of physico-chemical parameters were 40.45 g/l, 86.28 h, pH 7.9 and 30.29 °C for substrate concentration, HRT, pH and temperature respectively, predicting a yield of 57.73 ml H_2/g TVS on hydrogen. The experimental validation gave 58.62 ml H_2/g TVS, thus showing a 3.81% improvement on the optimized substrate.

4. Conclusion

A two-stage modelling and optimization of biohydrogen production on agro-municipal waste of BH, CS, OFSMW and the associated operational parameters was carried out. The study revealed that without a prior treatment of substrates, a high yield of biohydrogen could be achieved using optimized mixtures in the ratio of OFSMW:BH:CS = 30:0:0 or OFSMW:BH:CS = 15:15:0 with process operation at optimum setpoint conditions. An initial optimization of waste substrate mixture, followed by appropriate combinations of optimum operational variables enhances fermentation hydrogen production. These findings are of special interest for a large scale production of biohydrogen as the raw material is renewable, and no energy input is required for the substrate pretreatment, in addition to the environmental benefits.

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Semi-pilot scale production of hydrogen from Organic Fraction of Solid Municipal Waste and electricity generation from process effluents

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ABSTRACT

The production of hydrogen from Organic Fraction of Solid Municipal Waste (OFSMW) was studied on a semi-pilot scale. The potential of generating electricity using the process effluents was further assessed using a two-chambered Microbial Fuel Cell. A maximum hydrogen fraction of 46.7% and hydrogen yield of 246.93 ml H_2 g^{-1} Total Volatile Solids was obtained at optimum operational setpoints of 7.9, 30.29 °C and 60 h for pH, temperature and hydraulic retention time (HRT) respectively. A maximum electrical power density of 0.21 W m^{-2} (0.74 A m^{-2}) was recorded at 500 Ω and the chemical oxygen demand (COD) removal efficiency of 50.1% was achieved from the process. The process economics of energy generation from organic wastes could be significantly improved by integrating a two-stage process of fermentative hydrogen production and electricity generation.

1. Introduction

The effects of climate change, increased global demands for oil and natural gas are intensifying the search for alternatives to fossil fuels [1]. Hydrogen gas is an attractive future energy carrier due to its clean, efficient and renewable properties [2] and can be generated from various organic wastes. The feasibility of hydrogen production in dark fermentation with the Organic Fraction of Solid Municipal Waste (OFSMW) in laboratory scale experiments has been reported in various studies with yields of 76 ml g^{-1} VS [3], 122.9 ml g^{-1} COD [4] and 134 ml g^{-1} COD [5]. These were achieved under different optimal flask operational conditions. The industrial production of hydrogen from these wastes requires further understanding of the process dynamics at semi-pilot or large scale.

OFSMW is highly considered as substrate of choice for hydrogen production partly due to waste disposal problems and also its rich content of carbohydrate, biodegradability, and a high hydrogen potential [6,7]. South Africa generated 7.88 Mt of organic waste in 2011, and only 35% of these were recycled. The rest were mostly burnt or disposed on landfills [8]. Hydrogen production from these waste materials will not only contribute to sustainable energy but also assists to alleviate environmental hazards.

Hydrogen production from organic waste materials is more efficient, but much of the organic matter remains in solution. Current fermentation processes can only produce 2–3 mol

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H_2 mol^{-1} glucose, and results in 80–90% of initial chemical oxygen demand (COD) remaining in solution in the form of various volatile organic acids and solvents [9]. To improve the economics of hydrogen production from substrates, additional processes are therefore needed to recover the remaining energy [10]. Recently, there has been an upsurge of interest in using MFC technology for harnessing electricity generation from wastewaters and organic wastes while facilitating complete energy recovery and reducing the waste treatment costs [11,12]. MFCs are biochemical catalyzed systems that generates electrical energy through the oxidation of biodegradable organic matter in the presence of fermentative bacteria [13]. The bacteria present in the anode chamber of fuel cell generate electrons and protons, and the potential between the respiratory system and electron acceptor generates electricity. Hence, bacterial energy is directly converted to electrical energy. Protons migrate through a proton exchange membrane from anode to cathode [12]. MFC processes have been reported for an effective energy recovering from wastewater [9,14,15].

This work describes a semi-pilot scale production of hydrogen from OFSMW, then investigates the electricity generation potential from the process effluents using MFC.

2. Materials and methods

2.1. Hydrogen production in a semi-pilot scale reactor

2.1.1. Inoculum development

The hydrogen-producing mixed consortia was obtained from the anaerobic sludge collected from the Darvill wastewater treatment plant, Pietermaritzburg, South Africa. The sludge was heated at 100 °C for 30 min to deactivate the methanogenic bacteria, thus enabling the survival of hydrogen producing endospore-forming clostridia which were confirmed in our previous studies (unpublished results).

2.1.2. Substrate pre-treatment

Organic wastes were collected from food stores in Pietermaritzburg, South Africa and the OFSMW was simulated according to the method of Gomez et al. [16]. It was made up of 10% apple, 10% orange, 35% cabbage, 35% potatoes, 8% bread, and 2% paper. The total volatile solids content of OFSMW was determined according to Equation (1).

$$\text{Total Volatile Solids} = \frac{\text{Weight of dried waste} - \text{Weight of ash}}{\text{Weight of dried waste}} \times 100\% \quad (1)$$

2.1.3. Intermediate fermentation process phase

Prior to the pilot-scale process, an intermediate fermentation stage was carried out in a 1000 ml modified Erlenmeyer flask reactor, inoculated with 50 ml of pre-treated sludge. The reactor was fed with OFSMW at concentration of 40.45 g l^{-1}, supplemented with inorganic salts (in g l^{-1}): NH_4Cl 0.5, KH_2PO_4 0.25, K_2HPO_4 0.25, $MgCl_2.6H_2O$ 0.3, $FeCl_3$ 0.025, $ZnCl_2$ 0.0115, $CuCl_2$ 0.0105, $CaCl_2$ 0.005 and $MnCl_2$ 0.015. The working volume was made up to 500 ml with distilled water. Anaerobiosis was created by flushing the reactor with nitrogen gas for 3 min. The setpoints of initial pH, temperature and stirring speed were 7.9, 30.29 °C and 1.66 s^{-1} respectively and the process was carried out for 60 h.

2.1.4. Fermentation process

The semi-pilot hydrogen fermentation process was conducted in 10 L bioreactor (Labfors Infors HT bioreactor, Switzerland). Prior to use, the reactor was sterilized by autoclaving at 121 °C for 15 min. It was fed with 4500 ml medium of OFSMW and inorganic salts stated above, followed by inoculation at 10% with the previous 60 h intermediate culture. The temperature was controlled at 30.29 °C and the stirring speed was maintained at 1.66 s^{-1}. The initial pH of the reactor was adjusted at 7.9 with no further pH control. Anaerobiosis was created by flushing the reactor with nitrogen gas for 10 min through the gas sparger.

2.1.5. Process monitoring and analysis

The changes in the volume fractions of hydrogen and carbon dioxide of the evolving gas were continuously monitored using the F-Lab Biogas software previously described [17], running at 1 min sampling frequency and using the BCP-H_2, and BCP-CO_2 sensors (Bluesens GmbH, Germany). The measuring principle of the gas sensors was based on thermal conductivity detector and infrared technology, all with pressure compensation. The cumulative volume of these biogas was recursively software computed using their fractions in the evolving gas and the gas volume at each sampling interval according to Equation (2).

$$V_{H,i} = V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V_H(C_{H,i} - C_{H,i-1}) \quad (2)$$

where $V_{H,i}$ and $V_{H,i-1}$ are cumulative hydrogen gas volume at the current (i) and previous (i−1) time intervals, $V_{G,i}$ and $V_{G,i-1}$ the total biogas volumes in the current and previous time intervals, $C_{H,i}$ and $C_{H,i-1}$ the fraction of hydrogen gas in the headspace of the reactor in the current and previous time intervals, and V_H the total volume of headspace in the reactor [18].

The pH was monitored with a pH sensor (Mettler Toledo GmbH 405-DPAS-SC-K8S/325, Germany).

2.2. Electricity generation from process effluent using MFC

2.2.1. MFC structure and design

The MFC was constructed as described by Khan et al. [19] on a two-chambered design using glass material. The anodic and cathodic compartments were provided with inlets and sampling ports. A salt bridge made up of glass tube was used to connect the two chambers (length = 0.05 m, diameter = 0.012 m), and consisted of 10% agar, 5% KCl and 5% NaCl. The electrodes were made up of graphite rod (1.48 m^2 cross section), positioned at a distance of 0.05 m on either side of the salt bridge with equal projected surface areas of 2.19 m^2. Anaerobic conditions in the anode were achieved by sealing the flask with a rubber stopper. The cathode was operated under aerobic conditions. Prior to use, the electrodes were sterilized with 70% ethanol. The schematic diagram of MFC design is shown in Fig. 1.

2.2.2. MFC operation

The anodic chamber was fed with 630 ml of effluents from the semi-pilot scale fermentation bioreactor, and then inoculated with 70 ml of untreated sludge. Methylene blue (0.05 g l^{-1}) was used as a mediator in the anodic chamber. The cathodic compartment (700 ml working volume) was filled with 5% NaCl; air was continuously bubbled into the cathode for sufficient supply of dissolved oxygen within the medium. The pH of the effluent was adjusted to 7 using 10^3 mol m^{-3} sodium hydroxide. The anodic chamber was flushed with nitrogen gas (3 min) to create anaerobiosis. The outlet port of the anodic reactor was connected to a water displacement cylinder to collect the biogas (hydrogen, methane and carbon dioxide) produced during electricity generation. The experiment was conducted in duplicates at constant temperature (30 °C) using a water bath.

2.2.3. MFC analytical procedure and calculation

The voltage (V) in the MFC system was monitored and recorded every 3 h intervals using a digital multimeter (MDI10 Digital Multimeter, Major Tech, South Africa). For polarization, the voltage was recorded at varied external resistance from 75 to 2000 Ω connected for 15 min. The current (I), power (P), power density (PD), and current density (CD) were calculated according to Mohan et al. [20]. PD and CD were normalized to the anode surface area (2.19 m^2). The pH of anodic chamber was recorded daily using a bench top pH meter (Lasec, South Africa). The concomitant biogas produced during electricity generation was estimated according to Equation (2). The performance of MFC was also evaluated by assessing the COD removal efficiency during operation according to Equation (3). COD analysis was performed according to the standard methods [21].

$$\%\ \text{removal effeciency COD} = \frac{COD_i - COD_f}{COD_i} \times 100\% \qquad (3)$$

where COD_i and COD_f represents the influent and effluent COD concentrations (g l^{-1}) respectively.

3. Results and discussion

3.1. Lag phase of hydrogen production

The volume fractions of hydrogen and carbon dioxide were continuously monitored. As shown in Fig. 2A, the hydrogen production started after 4 h of fermentation. This short lag phase is due to the rich carbohydrate content of the OFSMW and its various organic matter composition which make it easily accessible to mixed microbial cultures as earlier reported by Zhou et al. [5]. This substrate primarily consists of kitchen type of waste with low lignin content which ranges from 0.9 to 12% [22] as compared to agricultural waste residues which have a complex polymer structure. The duration of lag phase can also be affected by the operational parameters such as pH and temperature. Comparative studies showed that the lag phase times are shorter at alkaline and mesophilic conditions compared to acidic and thermophilic conditions. This is attributed to the fact that the cytoplasm of bacterial species has a higher pH and its metabolism is not disrupted by alkaline conditions [23]. However, lag phase times are longer under acidic conditions due to disruption of cell's metabolism. Therefore bacteria have to induce acid tolerance response mechanism [24]. It has been reported that the activity of hydrogenase enzyme is inhibited by the low pH [25]. In laboratory flask experiments, lag phase times of 2.4, 4.8 and 14 h have been reported with substrate of lettuce, potato and rice respectively [3]. These substrates are easily hydrolyzed by hydrogen producing bacteria due to their biodegradable nature. In contrast, Lee and Chung [26] reported a relatively longer lag phase time of 24 h in a two-stage pilot scale process with 150 L working volume of hydrogen production using food wastes under near similar operational conditions, and this was attributed to the nature and composition of substrate. In addition, factors such as the

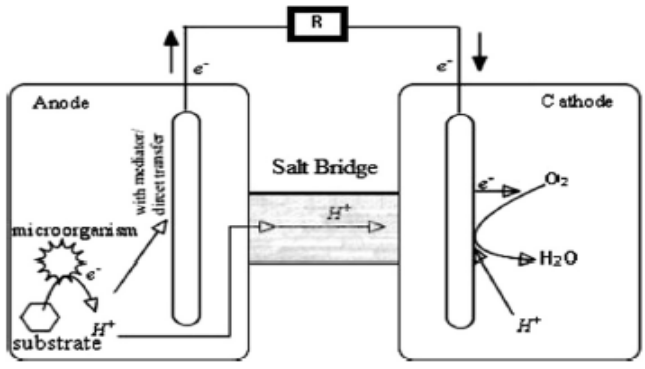

Fig. 1 – Schematic diagram of the microbial fuel cell used.

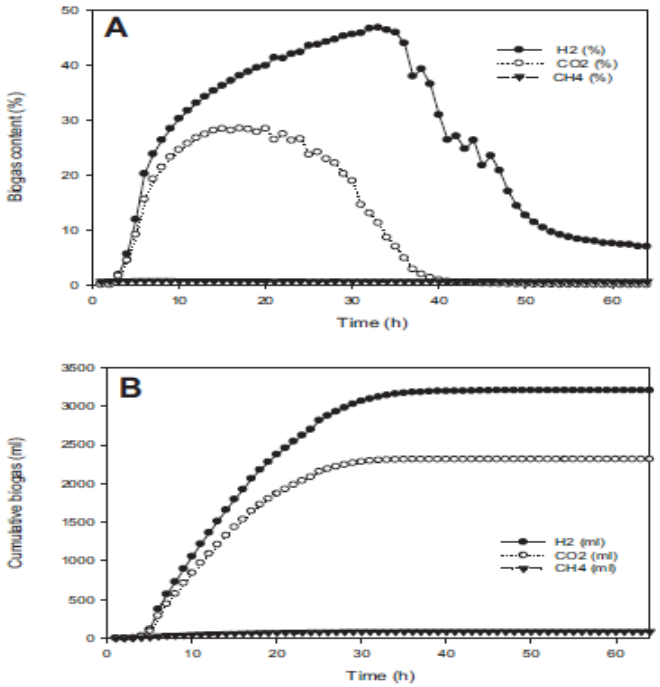

Fig. 2 – Evolution of biogas fractions of hydrogen, methane and carbon dioxide (A), and (B) the trends in cumulative biogas during a semi-pilot continuous monitoring.

reactor configuration and volume size affect the partial pressure and heat transfer within the reactor in pilot scale processes and hence the lag phase duration for hydrogen fermentation process is affected. A longer lag phase times observed in pilot scale studies from organic wastes may be due to practical engineering aspects such as the size and design of the reactor which affects parameters such as mixing, heat transfer and partial pressure in large scale fermentation processes. The results obtained in this study with a lag phase time of 4 h are in line with reported findings of hydrogen production of 0.1–3.6 h [7] and of 0.05–4.9 h [6] from food wastes in laboratory flask experiments at mesophilic conditions.

3.2. *Exponential and peak production phase of hydrogen*

The exponential phase of hydrogen production spanned from the process time of 4 h to about 32 h reaching a maximum hydrogen fraction of 46.5% and a cumulative hydrogen volume of 3118 ml (Fig. 2A and B). Zhou et al. [5] reported an exponential growth phase of 21.2 h (8.8 h–30 h) for anaerobic co-digestion of food waste and wastewater for hydrogen production in laboratory batch flask experiments. Hydrogen is produced during the exponential growth phase of clostridia in acidogenic process [27]. During this process, *Clostridium* species which are either proteolytic or saccharolytic organisms hydrolyze the substrate via acetate or butyrate fermentation reaction to produce hydrogen [25]. Spore germination and hydrogenase enzyme activation in hydrogen producing bacteria are observed during this process stage [28]. These have been reported as the most important factors in the overall hydrogen fermentation process [29,30]. The morphology of the prevailing hydrogen producing bacteria was observed using light microscope during this phase of fermentation (Fig. 3). Microbial population consisting predominantly of rod-shaped cells confirmed the presence of hydrogen producing clostridia within the bioreactor. Microbial community analysis of various hydrogen producing activated sludge systems showed that *Clostridium* species are dominant active hydrogen producers [31]. Their presence is reported to be more than 60% of total bacterial populations after pre-treatments [32]. Their dominance is possibly enhanced by the resistance of endospores [33].

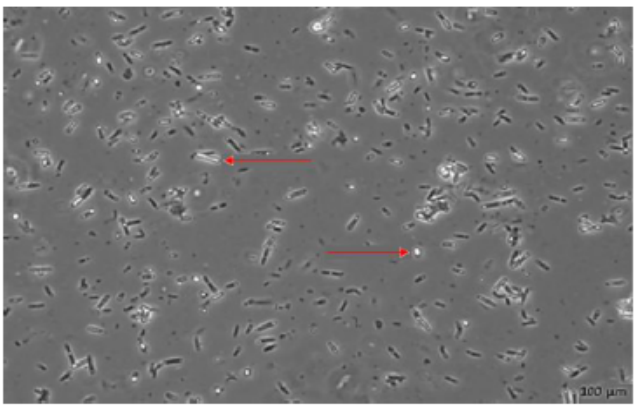

Fig. 3 – Morphology of hydrogen producing bacteria. Sporulating rod-shaped cells are indicated with an arrow.

The fermentation process showed a peak of hydrogen fraction of 46.7% with a cumulative hydrogen volume of 3139 ml at 33 h and lasted for 1 h. The duration of a steady peak hydrogen fraction depends on the substrate type and process conditions. For instance, Dong et al. [3] reported peak durations of 1, 1 and 3 h for potato, rice and lettuce respectively in laboratory flask processes at pH 5.5 and 37 °C. Whereas Lay et al. [34] observed a peak duration of 6 h in hydrogen production process from organic municipal waste under similar operational conditions. The reported peak of hydrogen fraction in semi-pilot scale varies with reactor size, process time and substrate used. For example, Lin et al. [35] using a 400 L bioreactor operated for 65 days obtained a peak in hydrogen fraction of 37.8% using sucrose medium and Chang et al. [36] using a 12 L bioreactor operated for 95 days obtained a peak value of 40.4% on molasses. With regard to process yield at semi-pilot scales, values of 1.04 mol H_2 mol^{-1} sucrose at 400 L [35], 2.91 mol H_2 mol^{-1} hexose at 20 L [37] and 1.40 mol H_2 mol^{-1} glucose at 12 L [36] have been reported. These observations point to the scale-dependent hydrogen production efficiency which might be due to traditional fermentation scale up challenges.

3.3. *Process decline phase*

A decrease in hydrogen fraction was observed from process time of 34 h–64 h and reached a minimum hydrogen fraction value of 6.9% (Fig. 2A). This can be attributed to the switch of fermentation process from acidogenic to solventogenic process as earlier reported by Khanal et al. [25]. Thus the change in process intermediates products from acetate, butyrate to acetone, butanol and ethanol or the acidogenic–solventogenic transition led to inhibition of hydrogen production. Hydrogen consuming bacteria such as homoacetogens can also pose a threat to hydrogen producers because these are versatile group of bacteria, strictly anaerobe, fast growing and endospore-forming organisms [32]. These bacterial species grow chemolithoautotrophically on hydrogen and carbon dioxide, producing acetate at higher hydrogen thresholds than methanogens or sulfate-reducing bacteria [25]. They have higher growth rates than other fermentative bacteria due to energy conservation from a combination of substrate-level phosphorylation and sodium-based chemiosmotic mechanisms [38].

3.4. *Carbon dioxide evolution*

The carbon dioxide production started from process time of 4 h and reached a maximum fraction of 28.4% and a cumulative volume of 1435 ml at 14 h (Fig. 2A). During this process time, a very high correlation (0.99) was observed between hydrogen and carbon dioxide evolution. This could be attributed to the acetate and butyrate fermentation pathways that generate 2 mol CO_2 mol^{-1} glucose. However, a steady carbon dioxide fraction of 28.4% was observed from 15 h to 24 h. It is likely that acetate fermentation was thermodynamically favored at this stage since it has a high theoretical yield of hydrogen (4 mol H_2 mol^{-1} glucose). Acetate and butyrate reactions are formed during dark fermentation processes but

their ratio varies with growth conditions [39]. Earlier studies by Van Andel et al. [40] showed that decreasing the partial pressure of hydrogen resulted in an increase in acetate/butyrate ratio and in turn enhances the hydrogen production.

3.5. pH evolution during semi-pilot fermentation process

A decrease in pH from 7.9 to 5.04 was observed during the first 4 h of hydrogen fermentation process (Fig. 4). In the previous studies, we reported a pattern of a sharp drop in pH at the late lag phase which was an early indicator for the onset of the log phase in dark fermentation process monitoring [17]. Hydrogen is associated with the production of volatile fatty acid (VFA) components such as acetate, butyrate and propionate [2]. The pH drop represents rapid production of VFAs within the medium [12]. From the process time of 10 h–46 h, the pH remained relatively stable within a range of 4.7 to 4.3 without the addition of a buffer. A similar observation has been reported by Zhi et al. [41] for a pH decrease from 7 to a relatively constant range of 4.65–4.85 in a non-buffered hydrogen production system. It is likely that this relative stability might be due to a balanced uptake of protons by hydrogenases according to Equation (4).

$$2H^+ + 2e^- \leftrightarrow H_2 \quad (4)$$

The control of pH during hydrogen fermentation remains necessary to prevent a possible metabolic shift and to suppress the hydrogen consumers while maintaining an enriched culture of hydrogen producing bacteria. pH control is more feasible at pilot scale using dedicated sensors and actuators [35,36] than in water bath shake flask systems. In the later, only the initial pH value is often reported.

3.6. Electricity generation using process effluent

Due to the traditional low yield of hydrogen generation on dark fermentation processes, the organic substrates in the effluent are not fully metabolized. A second bioprocess stage was adopted for further energy extraction using MFC. In addition the anodic chamber of MFC can operate as wastewater treatment reactor. The electrogenic bacteria used the suspended organic matters in the effluent for biomass development and electron generation. A gradual increase in MFC voltage was observed from 0.05 V to a maximum open circuit voltage of 0.48 V after 60 h of operation (Fig. 5A). Thereafter it showed a decreasing trend suggesting exhaustion of nutrients. During the MFC operation, the evolving gas from anodic chamber was analyzed with respect to hydrogen, methane and carbon dioxide. A cumulative hydrogen production of 9.2 ml was recorded. Operationally, hydrogen utilization occurs during electricity generation as protons move to the cathodic chamber, thus the observed volume of hydrogen evolved from anodic chamber is lower than the actual volume. These observations point to a feasibility of a concomitant generation of electricity and

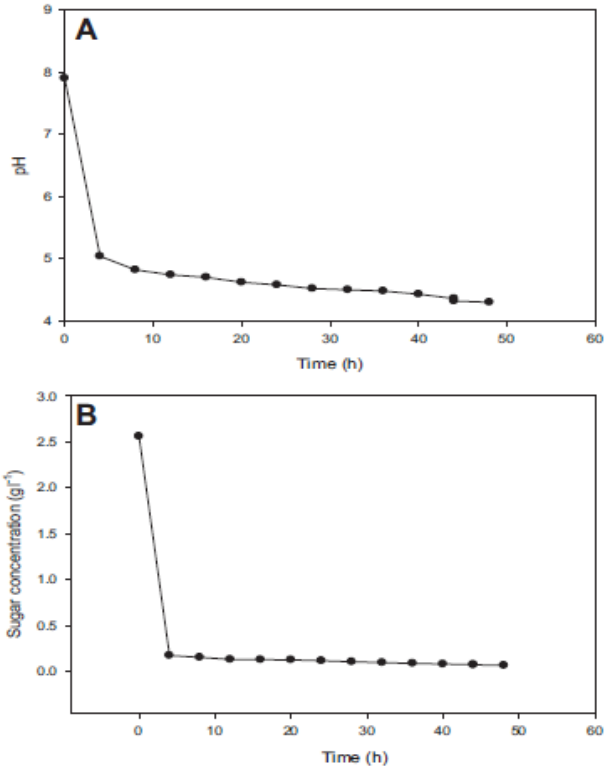

Fig. 4 – pH profile (A), and (B) sugar degraded by microorganisms during a semi-pilot continuous monitoring dark fermentation process.

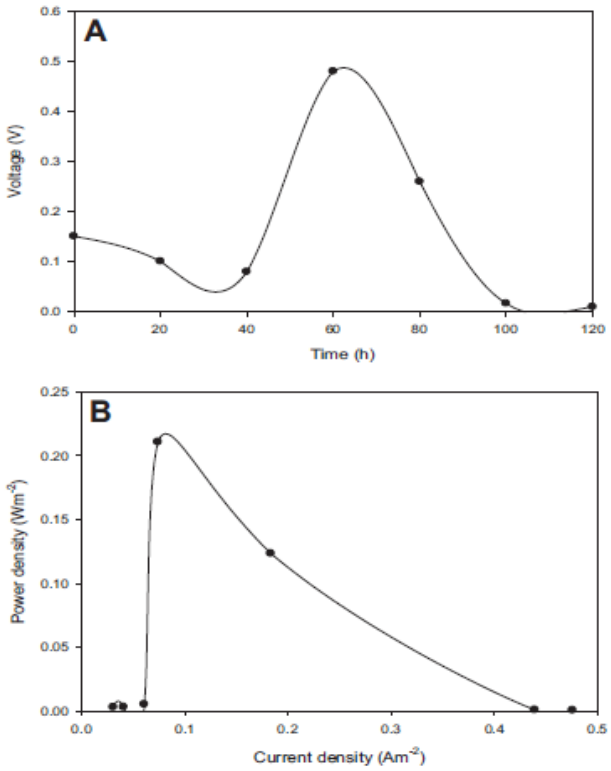

Fig. 5 – Electricity generation using fermented effluent from hydrogen producing reactor at 150 Ω (A), and (B) power density as a function of current density.

hydrogen. Niessen et al. [42] reported that hydrogen producing bacteria such as *Clostridium butyricum* and *Clostridium beijerinckii* were capable of producing electricity from starch.

The polarization sweep obtained by applying various external resistance helps to determine the operational point of the MFC. In practice, as the applied resistance becomes lower, there is a greater electron demands, forcing the microbial consortium to increase the metabolic activities, and in so doing improve the power and COD removal efficiency. This is sustainable if it is near the point of Maximum Power Transfer (MPT); obtainable from a polarization curve. It is usual practice to operate the MFC to the left side of power density peak, and at high voltage or low current density [12]. In this study the curve was obtained by plotting the calculated current density against the power density at various external resistance values. A maximum power density of 0.21 W m^{-2} (0.74 A m^{-2}) at 500 Ω was obtained (Fig. 5B). It is not feasible to directly compare the power output with other MFC processes in literature due to difference in operational setpoint parameters, surface area and type of electrodes, and different microorganisms used [43]. The construction of the MFC and concentration of organic matter also affects the generation of power outputs [13]. Oh and Logan [44] observed that generation of electricity in single-chambered process was 3.5 times higher than that achieved in two-chambered process, although a single-chambered MFC design has some challenges such as reverse polarization and low oxygen supply in the cathodic compartment. Some of the common electrogenic microbes with their associated maximum power densities are shown in Table 1. Electricity can be generated from diverse microorganisms particularly those microbes that are dominant in soil and wastewater samples (*Escherichia coli*, *Shewanell* species). MFCs can be operated using either pure or mixed cultures. Mixed cultures are more suitable for the use of complex substrates such as wastewater and biomass effluents, as single organisms generally metabolize quite a limited range of organic compounds [45]; as shown in Table 1, a higher power density of 5.85 W m^{-2} was obtained using mixed cultures.

The pH measurements over time during MFC operation showed that the anolyte pH decreased gradually from 7.2 to 4.21 (Table 2), due to production of fermentative metabolites which changed the buffering capacity of the medium. The trend of pH change was in line with active electricity generation in MFC processes [15]. The reported optimum pH in anodic chamber of MFC is in the range of 6–7 [43].

The MFC was also assessed on the COD removal potential of the anodic reactor. A decrease in COD concentration from 1.66 g l^{-1} to 0.83 g l^{-1} was obtained in the digesting effluent giving a COD removal efficiency of 50.1%. Butyrate and acetate which are the intermediate products of most fermentation are highly hydrolysable, and removal of 28.4–48.7% of acetate have been reported by Wang et al. [15] while Liu et al. [10] reported substrate removals of 98 and 99% for butyrate and acetate respectively. Cheng and Logan [11] reported that electricity could be produced in MFCs from acetate at yields approaching 99%.

These data highlight the feasibility of a concomitant generation of hydrogen, electricity coupled with an efficient COD removal using anaerobic fermentation of OFSMW.

Table 2 – Characteristics of the fermented effluent during electricity generation.

Parameter	Time (h)					
	0	24	48	72	96	120
pH	7.2	6.82	5.62	5.32	4.71	4.21
COD (g l^{-1})	1.66	–	–	–	–	0.83
Cumulative biogas (ml)	–	–	–	–	–	9.2
Hydrogen						
Carbon dioxide	–	–	–	–	–	5.2
Methane	–	–	–	–	–	3.8

–: Not available.

4. Conclusion

A semi-pilot scale hydrogen production process was carried out and the conversion of process effluents to electricity using Microbial Fuel Cell was assessed. The study revealed that a lag phase of 4 h, a peak hydrogen fraction of 46.7% and yield of 246.93 ml H_2 g^{-1} Total Volatile Solids were achievable at a semi pilot scale of dark fermentation using the organic fraction of solid municipal waste. Furthermore, electricity generation at a power density of 0.210 W m^{-2} and a chemical oxygen demand removal efficiency of 50.1% can be obtained from the process effluents using a two chambered membrane-less Microbial Fuel Cell. These findings highlight the feasibility of hydrogen scale up on organic fraction of solid municipal wastes, and a concomitant generation of electricity and COD removal from the process effluents. As the maximum theoretical yield of hydrogen production on pure glucose substrate is low (4 mol H_2 mol^{-1} glucose), further hydrogen scale up studies using the organic fraction of solid municipal wastes as substrate coupled with MFC for optimum bioenergy extraction would shorten the timeline for a more environmentally friendly and sustainable hydrogen economy development.

Table 1 – Maximum power densities in various studies of MFCs.

Microorganism	Reactor type	Substrate used	Power density (W m^{-2})	Reference
Digested sludge	Membrane-less MFC	Acetate	0.03	[15]
Escherichia coli	Single-chambered	Complex medium	0.60	[46]
Shewanella oneidensis	Miniature reactor	Lactate	3.00	[47]
Wastewater	Two-chambered	Acetate	0.37	[44]
Anaerobic sludge	Two-chambered	Inorganic salts	0.16	[12]
Wastewater	Single-chambered	Glucose	0.77	[14]
Corynebacterium MFCO3	Single-chambered	Glucose	7.30	[9]
Mixed cultures	Two-chambered	Glucose	5.85	[48]

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BIOTECHNOLOGICAL EQUIPMENT

FERMENTATIVE BIOHYDROGEN MODELLING AND OPTIMIZATION RESEARCH IN LIGHT OF MINIATURIZED PARALLEL BIOREACTORS

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ABSTRACT

In the last decade, there has been an upsurge of interest to make a transition from the depleting fossil-based energy sources to renewable ones. Fermentative biohydrogen has been repeatedly flagged as a potential future alternative energy carrier in recent publications. Research towards its scale-up requires accurate and high throughput optimization data on key process parameters. This has been hampered by conflicting findings, potentially owing to research procedures and bioreactor equipments used. This paper reviews the current state of fermentative biohydrogen optimization research on agricultural wastes, using miniaturized parallel bioreactors (MPBs). The monitoring and control of physicochemical parameters on these bioreactors is discussed and the prospect of enhancing biohydrogen process development with a novel featured parallel miniaturized bioreactor is presented.

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Keywords: miniaturized parallel bioreactors (MPBs), fermentation, biohydrogen production, bioprocess development

Introduction

Fermentative biohydrogen production is attracting increasing global attention owing to its non-polluting feature, low-cost and renewable source. Biohydrogen is a promising fuel for the future with many social, economic and environmental benefits to its advantage. It has a long-term potential to reduce the dependence on foreign oil and lower the carbon emissions from the transportation and the industrial sectors (45). It has a high energy yield of 122 kJ/g which is 2.75 times greater than its equivalent of hydrocarbon fuels (27), and its reaction with oxygen does not produce greenhouse gases such as carbon dioxide (CO_2). Biohydrogen production using dark fermentation is more feasible (65, 67, 77, 80, 82) because it generates very clean fuel hydrogen at an affordable cost. It has wide areas of application, e.g. as automobile fuel, as a source of distributed or central electricity, and for generation of thermal energy.

The achievement of higher yields is a critical research objective for the sustenance of biohydrogen as the fuel for the future. Biohydrogen productivities of 605 $mg \cdot h^{-1} \cdot L^{-1}$ by an undefined consortium is the highest productivity that has been reported so far (12). But this process is still not commercially viable (83). The maximum biohydrogen molar yield on glucose reported is 2.91 mol H_2/mol hexose (43). Besides, the production of hydrogen from glucose is too expensive to support economic H_2 production.

Research towards biohydrogen scale-up requires accurate and high-throughput optimization data on key process parameters, generated from multifactorial experimentation using state-of-the-art miniaturized and paralleled bioreactors. This paper reviews the current state of fermentative biohydrogen optimization research on agricultural wastes, using miniaturized parallel bioreactors (MPBs). The monitoring and control of physicochemical parameters on these bioreactors is discussed and the prospect of enhancing biohydrogen process development with a novel featured parallel miniaturized bioreactor is presented.

Biohydrogen production from agricultural wastes

Biohydrogen is produced from a wide variety of biomass substrates, including agricultural and forestry wastes, municipal solid wastes and animal wastes and residues (50, 70). Many agricultural and food industry wastes contain starch and/or cellulose which are rich in carbohydrates. The complex nature of these wastes may adversely affect their biodegradability. Starch-containing solid wastes are easier to process for carbohydrate and hydrogen gas formation (27). There are three obstacles to the economical production of glucose from cellulose-rich biomass: (i) most biomass is quite dispersed, making its collection costly, even though its intrinsic raw material price is low; (ii) the structure of cellulosic materials, with cellulose fibrils surrounded by hemicelluloses and then lignin, is difficult to penetrate; and (iii) the cellulose chain is difficult to break down to glucose and other sugars either chemically or enzymatically. The production of biohydrogen from crop waste biomass is limited by the hydrolytic activity of the microorganisms involved in the biological attack of the heterogeneous and microcrystalline structure of lignocellulosic component, and in the decomposition of cellulose-like compounds to soluble sugars.

Zhang et al. (85) reported a yield of biohydrogen of 57 $mL \cdot g^{-1}$ when cornstalk was treated with sodium hydroxide (0.5 % NaOH); this value was 19-fold higher than the yield obtained from untreated materials (3 $mL \cdot g^{-1}$). Furthermore, the authors investigated the production of biohydrogen from

cornstalk waste with mixed pretreatments of acid (0.2 % HCl) and heat, with a maximum yield of 150 mL·g^{-1}, or a 50 times increase as compared to the initial value, thus proving the efficiency of both acid and base pretreatment methods.

Wang and Jin (78) optimized fermentative biohydrogen production, using sugarcane molasses. The maximum biohydrogen yield obtained was 1.85 mol H_2/mol hexose; corresponding to a biohydrogen rate of 17.38 mmol·h^{-1}·L^{-1}. It was observed from these results that organic substrates rich in carbohydrate and protein content are suitable for maximum biohydrogen production. Kongjan et al. (30) investigated the optimization of biohydrogen production from wheat straw hydrolysate, using dark fermentation in both batch and continuously stirred tank reactors. The maximum hydrogen yield was 318.4 mL·g^{-1} at a hydrolysate concentration of 5 % (v/v) in a batch reactor. In the continuously stirred tank reactor, the hydrogen yield and production rate were 178.0 mL·g^{-1} and 184.0 mL·(day·$L_{reactor}$)$^{-1}$ (operating for 3-day HRT) respectively, corresponding to 12 % of the chemical oxygen demand (COD) from sugars. In another study, Chen et al. (10) enhanced biohydrogen production from untreated rice straw, using mixed cultures. The maximum cumulative hydrogen production, hydrogen production rate and hydrogen lag phase were 733 mL, 18 mL·h^{-1} and 45 h, respectively.

Appropriate pretreatment steps for the raw material are often required to enhance hydrolysis. The main pretreatments are based on mechanical, physical, chemical and biological techniques (48). A mechanical shredding step is essential to reduce particle size and increase the surface area of the organic waste prior to fermentation. As a consequence, solubility and fermentation efficiency are both favoured in the acidogenic fermentation process. Chemical pretreatment methods using oxidizing agents, alkali, acids and salts are most frequently reported because they require no direct energy input (48).

Strategies for improved biohydrogen production

Strategies for improving the biohydrogen production rate and yields have been based on genetic improvement of the microbial strains, or at the fermentative level, on the modelling and optimization of key process parameters, using response surface methodology or artificial intelligence techniques, or on inoculum and substrate pretreatment techniques.

Genetic manipulation of the production strains

Metabolic engineering involves the genetic modification of microorganisms to target and manipulate enzymatic, regulatory, or transport pathways that affect a particular microbial process (12). Recent success in genome sequencing and gene expression analysis has enhanced the ability to engineer microorganisms for specific metabolic tasks. Several studies show that H_2 production can be increased by directing the carbon flow toward synthesis of formate. Yoshida et al. (83) have experimentally proved that faster induction of the enzyme formate H_2 lyase (FHL) is possible by elimination of lactate and succinate formation. Increased yields from 1.08 mol/mol glucose to 1.82 mol/mol glucose in the *Escherichia coli* SR15 strain lacking lactate and succinate production have been achieved (83). Thus, by understanding which metabolic pathways contribute to and regulate the H_2 production, elimination of hydrogen-consuming reactions may be targeted to sustain and regulate the H_2 production rates. Detailed studies can be conducted to use genetic tools to overcome the metabolic barrier by manipulating the electron flux in H_2-producing organisms. The development of microbes that ferment multiple sugars, or which can directly utilize the naturally abundant sugars cellulose/hemicellulose can be targeted (12).

Applications of response surface methodology for determination of optimum process setpoints

Response surface methodology (RSM) has been widely used in various works to optimize the key parameters for enhanced biohydrogen production. Fermentation optimization based on a statistically planned experiment is a sequential process (7, 21). First, a large number of continuous factors are screened and insignificant ones are eliminated. The remaining factors could be optimized by response surface modelling. Finally, after model building and optimization, the predicted optimum is verified (68, 69). RSM designs are also useful for determining the interaction between the process variables important for the product yield. These include central composite design, mixture design, full factorial design and Box–Behnken design.

Ghosh et al. (16) used Box–Behnken design to optimize biohydrogen production, using substrate (glucose), fixed nitrogen, and light intensity during the single-stage photo-fermentation of glucose by the photosynthetic bacterium *Rhodobacter capsulatus*. They realized that the three independent variables, glucose, glutamate, and light intensity, had significant interactive effects on the biohydrogen yield and nitrogenase activity. The model has a coefficient of determination (R^2) of 0.99. The optimized biohydrogen yield shows an 85 % improvement (16).

Xing et al. (81) enhanced biohydrogen production from corn stalk by anaerobic fermentation, using central composite design. The optimum setpoints of the physicochemical process parameters were determined. A model with a coefficient of determination (R^2) of 0.96 was generated. Several studies on the optimization of fermentative biohydrogen production by the one-factor-at-a-time method have been reported. This strategy does not depict the interactive effect among the variables and does not guarantee the determination of optimal conditions (2, 53). In another study conducted by Liu et al. (38), the optimum conditions for biohydrogen production were predicted using response surface methodology when compost leachate was used as a source of nutrient for fermentative biohydrogen production. The model showed that the maximum cumulative biohydrogen volume (469.74 mL) and molar biohydrogen yield (1.60 mol H_2/mol glucose) could be achieved at 6174.93 mg/L glucose and 3383.20 mg COD/L leachate. A model with a coefficient of determination (R^2) of 0.8281 was generated.

TABLE 1

Miniaturized parallel bioreactors used for fermentative biohydrogen production

Bioreactor size (mL)	Working volume (mL)	Bioreactor parallelization	Substrate types	Reference
300–500	400	3 to 4	Peptone	Zabut et al. (84)
	450	16	Glucose and compost	Liu et al. (38)
	500	-	Glucose	Nath et al. (51)
250–300	250	-	Wheat starch	Oztekin et al. (56)
	285	-	Ground wheat	Argun et al. (2)
200–250	200	32	Food waste	Kim et al. (29)
	200	16	Glucose	Venkata Mohan et al. (74)
	200	8	Dairy wastewater	Venkata Mohan et al. (74)
	200	7	Rice slurry	Fang et al. (15)
150–180	150	-	Sucrose	Van Ginkel et al. (72)
	150	-	Wheat straw waste	Fang et al. (15)
	180	12	Market waste	Venkata Mohan et al. (73)
100–150	100	3	Apple pomace	Wang et al. (76)
	100	-	Glucose	Guo et al. (19)
	120	18	OFMSW	Lay et al. (35)
	125	27	Various metals	Lin and Lay (37)
50–100	50	-	Corncob	Pan et al. (57)
	50	-	Glucose	Oh et al. (54)
	50	-	Filtrate of biosolids	Wang et al. (75)
	50	12	Stale corn	Wang et al. (79)
	60	10	Mushroom waste	Lay et al. (33)
	60	4	Rice straw	Chen et al. (9)
	65	48	POME	O-Thong et al. (53)
	80	16, 4 and 50	Cellulose	Lay (34)
	40	-	Sucrose	Zhu and Béland (88)
	80	11	Lactose, cheese whey	Davilla-Vazquez et al. (13)
20–50	20	5	Xylose	Kongjan et al. (30)
	30	10	Glucose	Singh and Gu (66)
	30	5	Cassava and food waste	Zong et al. (90)
	34	5	Cassava and food waste	Zong et al. (90)
	39	6	Sucrose	Tao et al. (69)
<20	5	6 and 10	Zinc, iron and glucose	Ooshima et al. (55)

–: Not stated

These studies have shown that optimization methodologies are crucial for biohydrogen process development.

Artificial neural network in biohydrogen bioprocess development

Artificial neural network (ANN) is a type of artificial intelligence that is inspired by the way the brain processes information. It consists of simple synchronous processing elements called neurons which are connected to each other by links with their own weight factors (60). The network needs to learn the connection weights from an available training pattern in order to improve its performance over time. Various aspects have to be considered before a satisfactory neural network model is developed. The development of a neural network model includes database collection, analysis and pre-processing of data, design and training of the neural

network, test of the trained network and use of the trained neural network for simulations and predictions (41). Jo et al. (26) used ANN for maximizing biohydrogen production in a packed-bed bioreactor. The performance of the bioreactor was also predicted by the model on the key process parameters such as biohydrogen production rate and the metabolites in the effluent. Mu and Yu (49) used a neural network and genetic algorithm to predict the hydrogen production and the steady-state of an upflow anaerobic sludge blanket (UASB) reactor at various sucrose concentration and hydraulic retention times. Similarly, Guo et al. (20) estimated the biohydrogen yield and the chemical oxygen demand, using ANN in an expanded granular sludge bed (EGSB) reactor. ANNs are useful for prediction of biohydrogen production by their ability to learn complex non-linear input-output relationships, using sequential training procedures, and to adapt themselves to data (20, 39, 52, 64).

Lignocellulose substrate pretreatment strategies

Biological production of hydrogen from glucose is too expensive, and is thus not an economically viable process. Biohydrogen production from renewable sources such as agricultural biomass is economically feasible (39). Cellulose is the major constitute of plant biomass and highly available in agricultural wastes and industrial effluents, such as those from the pulp/paper and food industry (39, 58), and is significant for biohydrogen production. Initial pretreatment procedures are required to enhance the release of soluble sugars. Mechanical, physical, chemical and biological procedures are often adopted. Mechanical methods involve the breakdown of biomass residues into fine particles, thus increasing the specific surface area for subsequent hydrolysis. Physical treatments such as heating are extensively reported and have been shown to be more effective for disruption of cellulose structure, thereby enhancing the porosity of biomass residues and their accessibility to microorganisms during fermentation. However, this type of pretreatment is energy-consuming and does not remove the lignin content which withstands the enzymatic degradation (14, 87). Most of the chemical pretreatments that have been assessed to date (typically acid and alkali based methods) have the primary goal of enhancing the accessibility of biohydrogen-producing bacteria to cellulose by solubilizing the hemicellulose and lignin, and to a lesser degree decreasing the degree of polymerization and crystallinity of the cellulosic component and thus allowing biohydrogen-producing bacteria to have access to soluble sugars (42). Amongst these pretreatment technologies, acid pretreatment is considered to be efficient and easy to perform on industrial scale (57). Dilute-acid hydrolysis is widely reported as a method for pretreatment of lignocellulosic materials. Sulfuric acid and hydrogen chloride at concentrations below 4 wt % have been widely used, as they are inexpensive and effective. Dilute acid effectively removes and recovers most of the hemicellulose as dissolved sugars, and glucose yields from cellulose increase with hemicellulose removal to almost 100 % for complete hemicellulose hydrolysis (44).

Inoculum pretreatment methods

Heat-shock pretreatment methods have been widely applied to enrich biohydrogen-producing bacteria (34, 75, 87) and eliminate the non-spore-forming methanogenic bacteria, since hydrogen-producing bacteria, like most *Clostridium sp.*, can form protective spores under extreme conditions. Heat-shock treatment of hydrogen-producing mixed inoculum within a temperature window of 80 °C to 121 °C, and exposure time between 15 min and 120 min are commonly reported. Repeated heat-shock pretreatments and two-stage cultivation heat-shock pretreatment (87) have been reported in sucrose medium. Biohydrogen-producing seed has been obtained by treating the sludge by acid at a pH value of 2–4 (10, 64). Zhang et al. (86) applied the method of combined heat-shock and acid-shock on sludge for biohydrogen inoculum pretreatment. Cai et al. (8) has performed an extensive study on the pretreatment of sewage sludge by alkaline pretreatments and found that maximum biohydrogen occurred at initial pH of 11.

Miniaturized parallel bioreactors in bioprocess development

Miniaturized bioreactors

Bioprocess development for microbial cultivation and optimization are typically performed in expensive, mechanically complex and labour intensive, stirred-tank bioreactors (86). Therefore, microbioreactor technology has been used to address these challenges in order to reduce experimentation costs and speed up the research output. Industries have often employed simplified systems such as microtiter plates, shake flasks, test-tubes and spinner flasks for multi-factorial experimentation which offer ideal strategy to investigate the complex relationships between culture conditions and process outcomes (5, 36). Several authors have highlighted the need for miniaturized parallel bioreactors that monitors and controls the physicochemical parameters for high throughput experimentation (6, 7, 22, 24, 42, 59, 61). This is particularly important for multivariate experimentation. A microbioreactor must possess similar characteristics to a bench-scale bioreactor in terms of fermentation conditions, feedback control loops (18, 23, 31, 46), product quality and yield. Recently, some of these reactors have been enhanced with the capability to monitor and control parameters such as optical density (OD), pH, temperature and dissolved oxygen (DO) online and in real time and thereby avoid the need for sample removal (85). The optical sensor technologies have been applied to these bioreactors for online monitoring. Kensy (28) reported an online monitoring technique in continuously shaken microtiter plates (MTPs) for detecting the most relevant fermentation parameters such as biomass, fluorescent protein concentrations, pH and dissolved oxygen tension (DOT) in microbial fermentations with *Eschericha coli* and *Hansenula polymorpha* as model organisms. Earlier, Rivera (62) proposed a parallel microbioreactor with six wells, using optical sensors for monitoring and controlling cell culture conditions. A dissolved oxygen sensor based on the fluorescence quenching

of ruthenium diphenylphenanthroline dichloride and an optical sensor based on light transmittance were used in the six-well microbioreactor. These optical sensors were relatively inexpensive to fabricate and well suited for miniaturization and multiplexity.

Maharbiz et al. (40) integrated microtiter plate wells with silicon-monitoring technology in a 250 mL microbioreactor arrays with ion-selective field effect transistor (ISFET) sensors on a commercial printed circuit board. For aeration, oxygen was generated in the bioreactor by hydrolysis of water. The microbioreactor reported by Lamping et al. (32) was a scaled-down version of conventional stirred-tank bioreactors machined in Plexiglas and outfitted with air spargers and a stirring baffle.

Shake flasks are the most common miniature bioreactors and have been estimated to be used in over 90 % of all culture experiments across industry and academia for growing a wide range of microorganisms, e.g. bacteria (47), fungi (71), and yeasts (1) as well as mammalian cells (17). They are an inexpensive and effective way of reproducibly performing many types of industrially-relevant cell cultivations for process development (6). Shake flask bioreactors have various sizes ranging from milliliters to several litres. These vessels are made of glass or plastic materials, and are operated in a batch or fed-batch mode. The temperature is controlled using incubator or water bath, while the mixing is achieved through linear or orbital shaking. Non-baffled shake flasks can be operated such that bubbles are not formed which provides well defined gas-liquid mass transfer conditions (89). Generally, the pH is buffered or not, and poorly controlled.

Parallel bioreactors in bioprocess development

Automated parallel bioreactor systems performing several fermentation processes concomitantly can significantly speed up the development of biohydrogen production processes as well as other bioprocesses (4, 63). The high throughput of these systems leads to reduction in time, labour intensity, media cost, and space requirements, as compared to conventional bioreactors (4, 6). Different strategies have been proposed for parallel bioprocess development and optimization. Jo et al. (25) described the use of up to 48 stirred-tank parallel bioreactors for biohydrogen production. This approach involved gas-inducing stirrers for stirred-tank bioreactors on a 10 mL scale. To ensure an easy parallelization, a magnetic inductive drive was developed which allowed for the parallel operation of the 48 stirred-tank bioreactor in a bioreaction block. In this type of bioreactor, parameters such as pH, temperature and dissolved

TABLE 2

Monitoring and control of process variables in miniaturized parallel bioreactors for biohydrogen research

Bioreactor size (mL)	Control methods			H_2 sensing procedure	Reference
	pH	Temperature	Agitation		
300–500	pH probe	Water bath	Magnetic stirrer bar	GC (Hewlett Packard 5890, series II)	Zabut et al. (84)
	Initial pH adjusted	Water bath	Orbital shaker	GC (Perkin-Elmer,USA)	Nath et al. (51)
250–300	Initial pH adjusted	Incubator	Mixed manually	GC (Agilent, 6890)	Argun et al. (2)
200–250	pH probe	Temp regulated shaker	Orbital shaking	GC (Gow Mac, series 580)	Kim et al. (29)
	Initial pH adjusted	Temp regulated shaker	Orbital shaking	Microprocessor H_2 sensor (ATMI GmBH inc., Germany)	Venkata Mohan et al. (74)
	pH sensor and controller	Temp regulated shaker	Orbital shaking	GC (Hewlett-Packard 589011, USA)	Fang et al. (15)
150–180	Initial pH adjusted	Incubator	Orbital shaking	GC (Gow Mac, series 580)	Van Ginkel et al. (72)
	Initial pH adjusted	Temp regulated shaker	Orbital shaking	Microprocessor H_2 sensor (ATMI GmBH inc., Germany)	Venkata Mohan et al. (73)
100–150	Initial pH adjusted	Not stated	Magnetic stirring	GC (SP 6890)	Wang et al. (76)
	Initial pH adjusted	RCC	Rotatory shaking	GC (Shimadzu 8A)	Lay et al. (35)
	Initial pH adjusted	Incubator	Orbital shaking	GC (Model 310, SRI Instruments, Torrance, CA)	Baghchehsaraee et al. (3)
50–100	Initial pH adjusted	Incubator	Orbital shaking	GC (Agilent, 4890D)	Pan et al. (57)
	Initial pH adjusted	Incubator	No shaking	GC (Agilent, 4890D)	Wang et al. (79)
	Initial pH adjusted	Temperature regulated orbital shaker	Orbital shaker	GC(8700T, China)	Lay et al. (33)
	Initial pH adjusted	RCC	Rotatory shaking	GC (Shamadzu 8A)	Lay (34)
	Initial pH adjusted	Incubation room	Horizontal shaking	GC (Agilent 68090N, Germany)	Davila-Vazquez et al. (13)
20–50	Initial pH adjusted	Incubator	Orbital shaking	GC (Microlab, Arhus, Denmark)	Kongjan et al. (30)
	Initial pH adjusted	Incubator	Not stated	GC (Techcomp, 7900, China)	Zong et al. (90)
<20	Initial pH adjusted	Incubator	Not stated	GC (Producer not stated)	Ooshima et al. (55)

H_2: hydrogen gas; RCCS: rotatory cell culture system, OFSMW: organic fraction of solid municipal waste, GC: gas chromatography

oxygen were monitored and controlled online. The Sixfors benchtop device (Infors AG, Bottmingen, Switzerland) has six fermenters operating in parallel (6) and the Cellstation bioreactors (Fluorometrix Corporation, Stow, MA) allow 12 miniature stirred-tank bioreactors to be operated in parallel. Parameters such as pH and temperature are controlled online in these bioreactor systems, whereas agitation is achieved through baffles and impellers (85). The miniaturization and parallelization of bioreactors for biohydrogen is an attractive approach for development of this process.

Application of miniaturized parallel bioreactors in biohydrogen research

Multivariate fermentative biohydrogen research has been carried out in miniaturized parallel bioreactors. A working volume of 5 mL to 500 mL and bioreactor parallelization ranges from 3 to 50 have been used (**Table 1**). Various substrate types such as food, dairy, and agricultural wastes are used. A parallelization up to 50 bioreactors has been used with cellulose as a substrate and up to 32 bioreactors on food wastes. Although the parallelization level is correlated to the number of parameters under investigation rather than the nature of the substrate, the miniaturization scale is limited with the complexity of the medium, and glucose substrate for biohydrogen research has been used in a miniaturized bioreactor of a 5 mL working volume.

The monitoring and control strategies for the physicochemical parameters in these bioreactors for biohydrogen research are presented in **Table 2**. The pH value is either monitored with miniaturized pH probes and controlled by addition of acid or base, or not controlled and the initial pH value of the culture is adjusted at a desired setpoint, despite the fast drift in pH setpoints during the fermentation process as result of substrate types or metabolic activities. Temperature is regulated by water baths, incubators or shakers whereas agitation is achieved by magnetic stirrers or shaking water baths. In some cases, mixing is done manually at regular time intervals, however this method lacks consistency, reproducibility, efficiency and reliability.

In these reactors, the hydrogen fraction of the generated biogas is measured using real-time hydrogen sensors or gas chromatography which has the ability to measure other compounds produced during the fermentation process, but it has the traditional drawbacks of the class of problems associated with offline samplings. The cumulative hydrogen gas volume determined by gas chromatography is calculated according to Equation 1.

$$V_{H,i} = V_{H,i-1} + C_{H,i}\,(V_{G,i} - V_{G,i-1}) + V_H(C_{H,i} - C_{H,i-1}) \qquad \text{(Eq. 1)}$$

$V_{H,i}$ and $V_{H,i-1}$ are the cumulative hydrogen gas volume at the current (i) and the previous (i-1) time intervals, $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volumes in the current and previous time intervals, $C_{H,i}$ and $C_{H,i-1}$ are the fractions of hydrogen gas in the headspace of the bottle measured using gas chromatography in the current and previous intervals, and V_H is the total volume of headspace in the bottle (11).

Proposed features for novel miniaturized parallel biohydrogen bioreactors

Biohydrogen process development will inherently gain from bioreactor miniaturization and parallelization at least to understand the synergistic or antagonistic effects of multiple parameters' interaction on hydrogen yield and production rate. These reactors will need additional considerations on (i) parallelization, (ii) maintenance of the pH control setpoint, and (iii) real-time measuring of hydrogen fraction.

Parallelization

Production of biohydrogen is more economically feasible on multiple-waste substrate streams, incorporating several interacting key elements which are furthermore influenced by process physicochemical parameters. Multifactorial experimentation is thus required for process model development on these inputs.

Maintenance of the pH control setpoint

In most reported microbioreactors, the initial pH value of the culture is adjusted, with no further attempt to control. This variable does not remain constant, but drifts during the process, influencing metabolic fluxes, thus altering the yield and productivity data.

Real-time measuring of hydrogen fraction

To date, the offline gas chromatography analysis of the evolving fraction has been the prime procedure. Its shortcoming is an overestimation of the cumulative volume of the hydrogen biogas fraction, as the sampling interval increases. In our laboratory, cumulative biohydrogen volume of 135.60 mL and 157.61 mL was found while comparing two sampling intervals of 1 min and 12 h, respectively, on the same process (to be published elsewhere).

Conclusions

A critical challenge for hydrogen fermentation is the low hydrogen conversion efficiency (12). This may be overcome by using industrial, municipal or agricultural wastes which are abundant, costless and renewable. However, multivariate experimentations will be required to generate accurate fermentation information which is translatable into actionable intelligence for biohydrogen process scale-up. This requires novel bioreactor configurations with a high level of parallelization coupled with integration of on-line monitoring techniques for detecting the most relevant fermentation parameters in biohydrogen production. The development of micro-sensors is necessary in order to provide real-time and reliable bioprocess information and also to determine suitable parameter setpoints for maximum biohydrogen production.

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