Design, Implementation and Assessment of a Novel Bioreactor for Dark Fermentative Biohydrogen Production

by

Mariam Bibi Hassan Khan

(211553330)

Submitted in fulfilment of the academic requirements of

Master of Science

Discipline of Microbiology

School of Life Sciences

College of Agriculture, Engineering and Science

University of KwaZulu-Natal

Pietermaritzburg

South Africa

25 November 2020

PREFACE

The research contained in this dissertation/thesis was completed by the candidate while based in the Discipline of Microbiology, School Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was financially supported by National Research Foundation.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

Signed: Professor E.B. Gueguim Kana (Supervisor)

Date: 25 November 2020

DECLARATION 1: PLAGIARISM

I, Mariam Bibi Hassan Khan, declare that:

(i.) The research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;

(ii.) This dissertation has not been submitted in full or in part for any degree or examination to any other university;

(iii.) This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;

(iv.) This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) Their words have been re-written but the general information attributed to them has been referenced;

b) Where their exact words have been used, their writing has been placed inside quotation marks, and referenced;

(v.) Where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi.) This dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

(vii.) This dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

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DECLARATION 2: PUBLICATIONS

This thesis represents a compilation of published work and manuscripts where each chapter is an individual entity prepared as per the journals' specifications thus some repetition between chapters has been unavoidable. The first author (student) conducted all experimental work, data collection and manuscript preparation, under the guidance of the second author (supervisor).

1. Khan MBH, Gueguim Kana EB. Design, implementation and assessment of a novel bioreactor for fermentative biohydrogen process development. International Journal of Hydrogen Energy 2016;41:10136-10144. (Chapter 3)

2. Khan MBH, Gueguim Kana EB. Adaptation of cell Immobilization Cartridge unit to Continuous Stirred Tank Reactor Improves dark fermentative hydrogen production. Submitted to Renewable Energy. Under review. (Chapter 4)

Signed: Mariam Bibi Hassan Khan

Date: 25 November 2020

CONFERENCE CONTRIBUTIONS

1. Khan MBH, Gueguim Kana EB. Design, Implementation and Assessment of a Novel Bioreactor for Fermentative Hydrogen Process Development. School of Life Sciences Research Day. 20 May 2016. University of KwaZulu-Natal, Pietermaritzburg, South Africa. Poster presentation.

Signed: Mariam Bibi Hassan Khan

Date: 25 November 2020

Abstract

The majority of the world's energy consumption and electricity generation is derived from fossil fuel sources. Their consumption has a negative environmental impact, thus the need for renewable energies. Hydrogen being a high energy zero carbon fuel source presents a profound appeal. Hydrogen may be produced biologically via various methods, this work involves dark fermentative hydrogen production (DFHP). A review of literature on the physicochemical parameters affecting fermentative hydrogen bioprocess was conducted. Bioreactor design was identified as a fundamental component that regulates the overall process outcome and was therefore analysed at length. The review highlighted that existing reactor configurations are unable to sustain a comprehensive criteria of efficient DFHP. A consolidation of biomass retention and non-invasive agitation were distinguished as crucial. The need for a novel reactor configuration possessing these attributes was consequently accentuated.

This study focuses on the design, implementation and assessment of novel bioreactor configuration for DFHP. The vessel was formed from a 2L glass and fitted with ports. Three 3D-printed permeable cartridges enclosed immobilized microbial cells and functioned as baffles. The localization and motion of the cartridges promoted improved exposure between microbial cells and substrate. Agitation was accomplished by rocking the vessel at 180°. All the control set points were adjustable, presenting the option of evaluating diverse control regimes. The implemented reactor showed a 35% increase in the peak hydrogen fraction and a 58% reduction in lag time compared to the control shake flask reactor. These findings showed that the novel reactor configuration, by means of the cartridge structure supporting the immobilized cells, enhanced the biohydrogen production process.

Subsequently, a preliminary scale up of the cartridge concept was implemented and incorporated into a continuous stirred tank reactor (CSTR). The cartridge (46x40x300mm) consisted of perforated hollow rectangular tubes, joined to form a

single amalgamation. This unit was used as substitute for the standard impellers of the CSTR and aligned at 120° laterally to the agitating shaft. The modified reactor prepared with Immobilized cells in cartridge (ICC) was comparatively assessed with the standard CSTR operated with suspended cells in reactor (SCR) and immobilized cells in reactor (ICR). ICC reduced fermentation time by 52 and 65% compared to SCR and ICR respectively. Gompertz model coefficients indicated a 98 and 37% increase in the maximum hydrogen production rate (Rm) using the ICC compared to the SCR and ICR fermentations respectively. ICC also showed better pH buffering capacity and complete glucose degradation. These findings further demonstrated that the scale up reactor configuration with the cartridge structure improved biohydrogen productivity, yield and process economics.

The novel configuration reduced process time, improved Hydrogen yield and ensured complete substrate degradation. Furthermore, the structural integrity of immobilized cells was maintained. These findings demonstrated that the novel bioreactor design improved biohydrogen production and showed potential for further DFHP research and development.

Keywords: Novel biogas bioreactor, Dark fermentation, Biohydrogen, 3D Printing, Cell immobilization, CSTR.

Acknowledgements

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

I would like to thank:

My Mother for her patience and understanding, my brothers for accompanying me on midnight sampling runs and my sister for earnestly listening to all my ramblings.

Professor Kana for giving me the opportunity to do this MSc, entrusting this project with me and providing the tools and equipment that was necessary.

The National Research Foundation for funding this research.

Professor Theresa HT Coetzer, Dr Robert D Stone, Dr Charles Hunter, Dr Sumaiya Jamal-Ally and Professor Carola Niesler for the opportunities that they have provided.

My lab and department mates Selisha, Yeshona, Preshanthan, Isaac, Dr Faloye, Kimberley, Gabriel, Riesha, and Daneal for making lab 117 the place it is.

Ashrenee, Lorika, and Cynthia for their encouragement, assistance and advice.

The technical and admin staff of the Microbiology Department, Miss Diane Fowlds, Wilsonn Sikhakhane, Natalie Jones and Sibongile Ntuli for their dedication and assistance.

I would also like to acknowledge the advice, patience and dedication displayed by the UKZN glass blowing team, the mechanical workshop management and technical staff as well George Carelse.

An extra special Thank You to Dr Charles Hunter and Celeste Clarke, who were there for me when I needed it the most, whose assistance and advice I will never forget.

I would like to dedicate this work to the people of Palestine, my inspiration.

ﷺ our Prophet, our Honour.

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List of Abbreviations

$(NH_4)_2SO_4$	Ammonium sulfate
3D	Three-dimensional
3-D	Three-dimensional
BBC	British Broadcasting Corporation
BP	Beyond Petroleum
$C_6\ H_{12}\ O_6$	Glucose
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate
CFD	Computational fluid dynamics
$C_{H,i}$	Fraction of hydrogen gas in the headspace of the bioreactor current interval
$C_{H,i-1}$	Fraction of hydrogen gas in the headspace of the bioreactor previous interval
CH_3COOH	Acetate
CO_2	Carbon Dioxide
COD	Chemical oxygen demand
C_P	Control parameters (chapter 3)
C_P	ICC parameters (chapter 4)
CSTR	Continuous Stirred Tank Reactor
DFHP	Dark Fermentative Hydrogen Production
e	2.718
EPS	Extra polymeric substances
exp	Exponential
FBR	Fluidized bed reactors
$FeCl_2 \cdot 4H_2O$	Iron dichloride tetrahydrate
g	Gram
GAC	Granular activated carbon
h	Hour
H(t)	Cumulative volume of hydrogen production (ml)

H_2	Hydrogen
H_2O	Water
HPP	Hydrogen Partial Pressure
HRT	Hydraulic retention time
HY	Hydrogen yield
ICC	Immobilized Cells in Cartridge
ICR	Immobilized Cells in Reactor
IPCC	Intergovernmental Panel on Climate Change
KH_2PO_4	Potassium dihydrogen phosphate
kJ	kilojoule
L	Litre
M	Molarity (moles of solute per litre of solution)
MBR	Membrane bioreactor
min	Minutes
ml	Millilitre
mm	millimetre
$MnCl_2 \cdot 4H_2O$	Manganese(II) chloride tetrahydrate
$Na_2MoO_4 \cdot 2H_2O$	Sodium molybdate dihydrate
NASA	National Aeronautics and Space Administration
$N_{g,f}$	Final glucose concentration
$N_{g,i}$	Initial glucose concentration
$N_{H,F}$	Cumulative hydrogen volume
NRF	National Research Foundation
P	Hydrogen production potential (ml)
PBR	Packed bed reactors
PLA	Polylactic acid
PLC	Programmable Logic Controller
psa	Pressure swing adsorption
PVDF	polyvinylidene fluoride
r^2	Coefficient of determination
RITA	Recipient for automated temporary immersion
R_m	Maximum hydrogen production rate (ml h^{-1})
R_P	Reactor parameters (chapter 3)

R_P	SCR or ICR parameters (chapter 4)
rpm	Revolutions per minute
s	Seconds
SCR	Suspended Cells in Reactor
SEM	Scanning electron microscopy
spp.	Species pluralis (Latin for multiple species)
SRT	Solids retention time
t	Incubation time (h)
TIS	Temporary immersion systems
UASB	Up-flow anaerobic sludge blanket
VFA	Volatile fatty acid
$V_{G,i}$	Total biogas volumes current time interval
$V_{G,i-1}$	Total biogas volumes previous time interval
V_H	Total volume of the headspace in the bioreactor
$V_{H,i}$	Cumulative hydrogen gas volume current time interval
$V_{H,i-1}$	Cumulative hydrogen gas volume previous time interval
$ZnSO_4 \cdot 7H_2O$	Zinc sulfate heptahydrate
λ	Lag-phase time (h)

Chapter 1

General Introduction

1.1. The need for renewable energy sources

Majority of the world's fuel originates from fossil sources, whether natural gas, oil or coal. According to the annual BP statistical review (2020) the global proved reserves of oil is sufficient to meet 50 years of global production. The global power generation also relies mainly on fossil fuels, most of the world primarily uses coal and natural gas followed by hydroelectricity (Figure 1). This year for the first time, renewable energy has surpassed nuclear energy (BP, 2020).

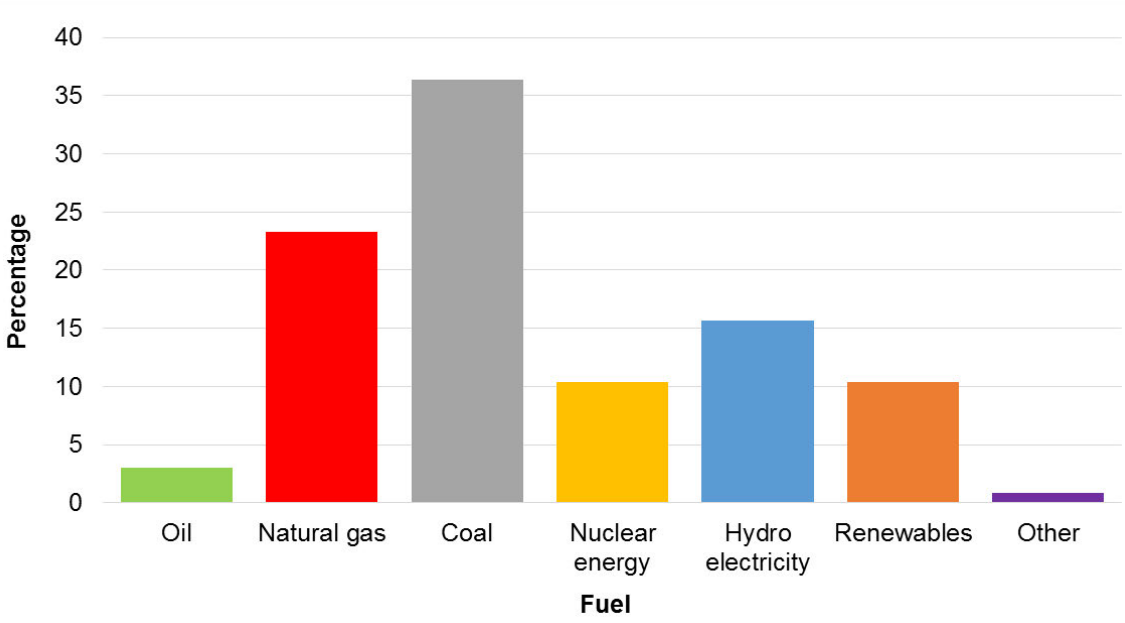

Figure 1: Share of global electricity generation by fuel (BP, 2020)

Different regions of the World utilise different fuels for electricity generation according to accessibility and natural resources. Hydroelectricity dominates central and south America whilst natural gas is mainly used in the Middle East and the commonwealth of independent states (Figure 2). Nuclear energy is quite common in Europe even though it is closely followed by renewables (Figure 2), coal forms 58% of the Asia

Pacific's electricity generation (Figure 2). Even though natural gas is cited as Africa's largest source of electricity, South Africa mainly uses coal for this purpose (BP, 2020). North, South and Central America dominate the World's biofuel production, grossing more than 67% (BP, 2020).

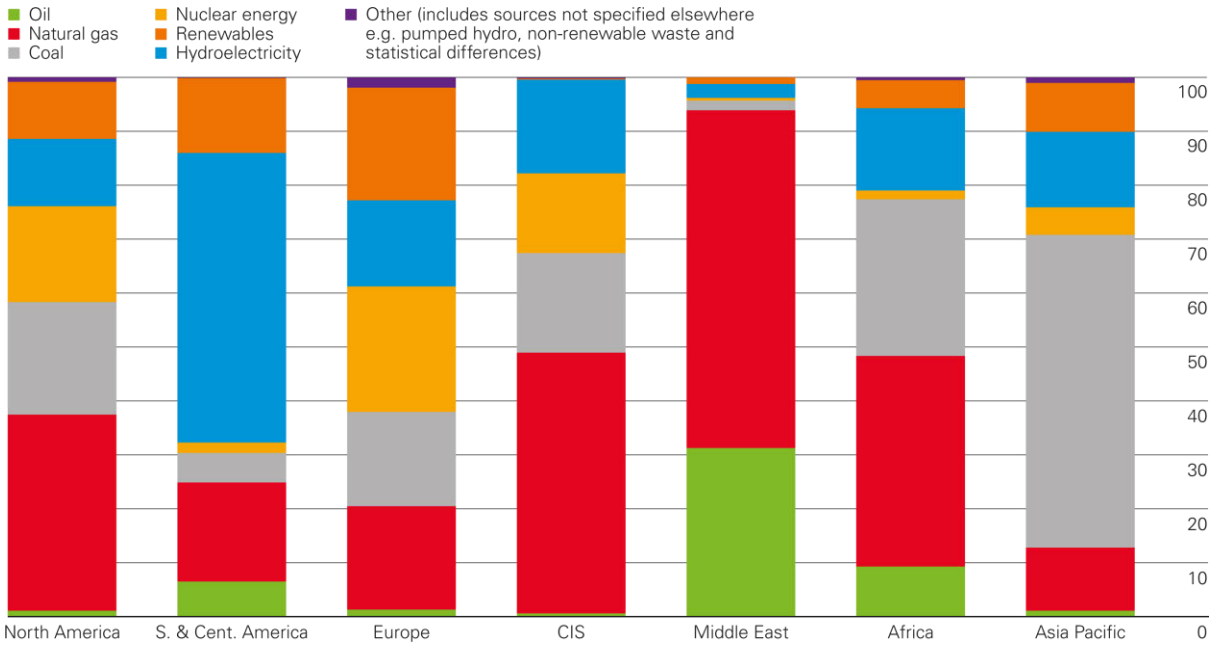

Figure 2: Regional electricity generation by oil, natural gas, coal, nuclear energy, renewables, hydroelectricity and other (adapted from BP, 2020)

According to the Intergovernmental Panel on Climate Change (IPCC), (Hoegh-Guldberg *et al*., 2018) human induced global warming has intensified temperatures in land and ocean regions. A 1.5°C increase in global temperature was reported compared to the pre-industrial period (Hoegh-Guldberg *et al*., 2018). Marine environments have shown an increased frequency and duration of marine heat waves causing changes in environmental habitats as well as other natural and human systems (Hoegh-Guldberg *et al*., 2018). Fossil fuel derived energy sources are the key reason for environmental decline and a salient form in global warming, greenhouse gas emissions and depletion of the ozone layer (Baldwin and Lenton, 2020; Last, 1993). Repercussions of global warming include an increase in the number of heat wave days, high fire danger days and reduced soil moisture availability (Engelbrecht *et al.*, 2015). Monitored temperatures within the sub tropics and central tropics of Africa

over a period of 5 decades show a rise in temperature of greater than two times the global rate (Engelbrecht *et al.*, 2015). Cumulative effects of environmental change could destabilize the economy of Africa as there are millions of farmers that rely on natural rainfall for production of crops used locally and imported to increase revenue. South Africa has suffered its driest years in recorded history during 2015 and 2016, the average rainfall in 2015 was 403mm compared to the years between 1904 and 2015 which was 608mm (Stoddard, 2016). The South African citrus export industry, second largest in the world, was impeded as a result of water restrictions, thus, jeopardising a multi-billion-rand trade (Stoddard, 2016). The meteorological office of the United Kingdom has predicted that the year 2020 will witness one of the greatest surges in atmospheric carbon dioxide since the start of record keeping (Baynes, 2020). The drastic increase was attributed to the ongoing devastating wildfires in Australia, which has burned over sixteen million acres during its three month reign until the end of January 2020 (Tarabay, 2020). These fires not only increase atmospheric CO_2, but burns through vegetation which would customarily soak up CO_2 (Baynes, 2020). Inception of this devastating cycle of destruction lays at the door of human induced global warming, and was supplemented by the El Niño events, which caused drastic temperature fluctuations globally (Baynes, 2020). It was inferred that the El Niño events contributed to the drought in South Africa, flooding in South America and intensive hurricanes in the eastern tropical pacific (Buis, 2015). In addition to the considerable negative effects that fossil fuels have on the environment, the source of majority of the world's oil reserves is beset by war and instability. Fluctuations in cost are prevalent as a result of attacks on tankers transporting oil as well as the recent drone attacks on various production facilities (Marcus and Prescott, 2019). This further reinforces the need for alternative energy sources.

1.2. Hydrogen as an alternative energy source

Hydrogen presents immense potential as a clean and renewable energy source, as it has the highest gravimetric energy of any known fuel (Levin *et al.*, 2004). Researching techniques to enhance the production, distribution and storage of hydrogen is required to improve economic feasibility (Dunn, 2002). Furthermore hydrogen is congruent with electrochemical and combustion processes to convert energy in a manner that does participate in environmental pollution and climate change via carbon based emissions (Levin *et al.*, 2004). Ausubel (2000) stated that "The trend toward "decarbonization" is

the heart of understanding the evolution of the energy system". Biological hydrogen production can be carried out via three primary methods; photodecomposition of organic composites, biophotolysis of water, and fermentation of organic wastes (Hallenbeck and Ghosh, 2009). Fermentation of organic wastes occurs at a higher rate, utilizes waste materials and possesses the potential to be integrated with other processes (Chandrasekhar *et al.*, 2015; Guo *et al.*, 2010; Kumar *et al*., 2017). The highest theoretical yield of hydrogen is 4 mol H_2 mol^{-1} glucose with 2 mol acetate as a by-product (O-Thong *et al.*, 2019), as shown below.

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

Furthermore dark fermentative hydrogen production (DFHP) can serve the dual purpose of producing clean energy and treating organic waste (Lalit Babu *et al.*, 2009), and this may be achieved at a cheaper cost than other methods (Waligórska, 2012). The process is however governed by various physicochemical factors and may be restricted by decreased hydrogen yields (Nath and Das, 2004; Waligórska, 2012). These factors include temperature, pH, hydraulic retention time (HRT), hydrogen partial pressure, inoculum type, preparation and state, substrate type amongst other physicochemical influences (Jung *et al.*, 2011). Bioreactor configuration and operation directly impacts the microenvironment of the reactor, such as hydrodynamic performance, microbial population and the mass transfer between substrate and inoculum (Cresson *et al.*, 2008; Jung *et al.*, 2011; Venkata Mohan *et al.*, 2007).

1.3. Research motivation/Problem statement

Bioreactors are central to fermentation processes and they provide a regulated microclimate enabling peak cellular growth and/or product formation (Najafpour, 2007). Batch mode of operation was recommended as it retains higher suitability for initial optimization studies (Balachandar et al., 2013; Davila-Vazquez et al., 2008; Hallenbeck and Ghosh, 2009; Kapdan and Kargi, 2006; Saratale et al., 2019). At a laboratory scale, shake flask reactors in shaking waterbaths or rotary shakers are commonly used for DFHP (Chen *et al.*, 2015; Mandal *et al.*, 2006; Lo *et al.*, 2008). During process scale up, continuous stirred tank reactors (CSTR) are employed (Chu *et al.*, 2016, Zhang *et al.*, 2006, Salem *et al.*, 2017). These standard configurations are plagued with various challenges for biohydrogen research and production. The

shake flask reactor is of a simple design and widely available, however it has poor gas and mass transfer resulting from inefficient agitation (Kennedy and Krouse, 1999). Considering the importance of laboratory scale studies, it is vital to generate accurate and reliable process data from the earliest possible stage. The CSTR has various advantages including highly effective mixing, thus advanced mass transfer (Chu *et al.*, 2016; Balachandar *et al.*, 2013; Saratale *et al*., 2019), however, this comes at a cost of excessive sheer stress and biomass washout. The CSTR is incapable of maintaining immobilized microbial cells (Balachandar *et al.*, 2013; Saratale *et al*., 2019). Bioreactor configurations capable of handling immobilized cells have shown a low efficiency for heat and mass transfer, reduced substrate conversion and decreased hydrogen production (Show *et al.*, 2011; Balachandar *et al*., 2013; Saratale *et al*., 2019). Standard reactor configurations that are available are inadequate for DFHP as they are unable to meet the complete criteria of efficient DFHP. A specialized reactor design could improve hydrogen yield and process stability (Hallenbeck and Ghosh, 2009). It would benefit the design to incorporate biomass retention capabilities with high mass transfer in order to optimise product formation (Najafpour, 2007).

1.4. Aims and objectives

The aim of this study was to design a novel bioreactor configuration for fermentative biohydrogen production, implement the design into effect and assess its potential compared to existing bioreactor configurations.

The following detailed objectives were undertaken:

i. A laboratory scale 2 L bioreactor optimized for heat and mass transfer, and biomass retention was designed using OpenSCAD software.

ii. Internal structures of the 2L reactor were 3D-printed in the laboratory using Polylactic acid (PLA) filament in a REPRAP 3D printing machine.

iii. The 2L laboratory reactor was comparatively assessed with a shake flask reactor of same volume at standard operational process conditions.

iv. Based on the initial configuration above, a 13L semi pilot scale prototype was designed and implemented using a modified CSTR.

v. The semi pilot prototype was comparatively assessed with a standard CSTR under uniform conditions of temperature, inoculum concentration and process pH.

1.5. Outline of dissertation/Thesis structure

This dissertation contains five chapters presented in research paper format, each chapter is self-contained, comprising an introduction, materials and methods, results and discussion, conclusion and references. Bioreactor design and assessment are pivotal to all chapters.

Chapter 2 discusses a literature review of physicochemical parameters affecting dark fermentative biohydrogen production. Bioreactor configurations used in biohydrogen production are described. Their advantages and limitations are detailed.

Chapter 3 focuses on the design, fabrication and assessment of a novel laboratory scale bioreactor. The novel configuration was compared experimentally to a shake flask reactor for dark fermentative hydrogen production.

In Chapter 4 a semi pilot scale prototype was developed based on the results achieved at a laboratory scale (Chapter 3). The design of the cartridge concept was modified and incorporated into a continuous stirred tank reactor (CSTR), the resulting hybrid was assessed in comparison to a standard CSTR.

The final chapter, Chapter 5 integrates aspects of the study and provides concluding remarks and future suggestions pertaining to the novel bioreactor designs formulated herein.

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Chapter 2

Review of physicochemical parameters and bioreactor configurations influencing dark fermentative biohydrogen production

2.1. Abstract

This review provides a synopsis of the physicochemical parameters effecting dark fermentative hydrogen production. Microbial inoculum, substrate type, hydrogen partial pressure, temperature, pH, hydraulic retention time and volatile fatty acids production were identified as being key to the chemical processes. Various bioreactor configurations commonly used for dark fermentative hydrogen (such as the continuous stirred tank reactor, membrane reactor, packed and fluidized bed reactors, and the up-flow anaerobic sludge blanket reactor) are examined and the pros and cons of each highlighted. In addition, the prospect of configurations not commonly used for hydrogen production is discussed. The potential of enhancing biogas production by developing a novel bioreactor design is examined

2.2. Introduction

The rapid depletion of fossil fuel reserves and the adverse environmental impact associated with their use necessitate renewable and non-toxic fuel sources (Chopra et al., 2020). Hydrogen has a comparatively high energy yield, amounting to 122kJg^{-1} which is 2.75 times more than hydrocarbon fuels (Kapdan and Kargi, 2006). There are no adverse side effects or harmful by-products formed upon hydrogen combustion, as only water is produced (Nath and Das, 2004). Three approaches for biological hydrogen production have been reported. Photodecomposition of organic composites, biophotolysis of water and fermentation of organic wastes (Hallenbeck and Ghosh, 2009). Photodecomposition and biophotolysis depend primarily on solar energy and require expensive photobioreactors (Hallenbeck and Ghosh, 2009). Hydrogen

production via fermentation of organic materials is independent of light and utilizes waste materials (Nath and Das, 2004; Lalit Babu *et al*., 2009), thus serving a dual purpose. This approach has the potential to reduce the cost of waste disposal and convert the waste material to a high value product. Sustainability and self-sufficiency in dark fermentative hydrogen production (DFHP) may be enhanced by means of merging the optimum parameters of various experimental studies to streamline the overall process. Physicochemical conditions that need to be optimised include inoculum type, preparation and state; temperature; hydraulic retention time (HRT); hydrogen partial pressure; pH; substrate type and bioreactor configuration (Jung *et al*., 2011). Continuing research of these factors contributes to our knowledge of DFHP and helps to optimize the process (Fang and Liu 2002; Venkata Mohan *et al*., 2007a; Guo *et al*., 2010; Chong *et al*., 2009; O-Thong *et al*., 2009; Lee *et al*., 2006; Fan *et al*., 2006; Zhang *et al*., 2006; Mandal *et al*., 2006; Mizuno *et al*., 2000).

Fermentation substrates and microbial inocula are selected based on availability and cost. Mesophillic temperatures are generally preferred to reduce the energy input and pH is determined by the choice of inoculum. Hydraulic retention time, mass transfer and hydrogen partial pressure are factors that affect all biohydrogen processes. Short hydraulic retention times promote increased hydrogen production (Jung *et al*., 2011). HRTs of between 8-12 hours was suggested for liquid substrates (Hawkes *et al*., 2002) although times as short as 6 hours were previously used with added benefits (Zhang *et al*., 2006). Microbial cell immobilization allows microbial growth independence over hydraulic retention time (Wu *et al*., 2006; Hallenbeck, 2009; Hallenbeck and Ghosh, 2009). This allows for biomass retention in dynamic systems running continuous processes. The effects of mass transfer and hydrogen partial pressure are similar to those of agitation. Bioreactor configurations impacts on cell growth and product formation. Various features of a bioreactor impact the chemical processes that occur within, for example, the agitation of a reactor directly influence the state of homogeneity and mass transfer the reactor is able to maintain. Agitation indirectly affects the hydrogen partial pressure (Kraemer and Bagley, 2007), as the rate of agitation plays a structural part in the transfer of metabolic gasses across the liquid to gas phase (Hawkes *et al*., 2007). Continued expulsion of Hydrogen enables this gas to exit the reactor and reduce the Hydrogen partial pressure, which when accumulated inhibits further hydrogen production. The bioreactor design is fundamental, as it

impacts on cell physiology and product formation. This chapter aims to give a basic overview of the factors that affect biohydrogen production with special emphasis on existing bioreactor configurations and their favourable attributes.

2.3. Impact of physico-chemical factors on biohydrogen production

2.3.1. Type of microbial inoculum

The type of microorganisms used impacts the hydrogen yield, its purity and the cost of the operation (Ntaikou *et al*., 2010). Aseptic conditions are necessary when pure cultures are used, complicating the design of the system. A higher hydrogen production efficiency and reduced by-product formation are benefits of pure cultures (Waligórska, 2012). Such systems have higher cost indications and are highly susceptible to contamination over long-term operation. The use of mixed cultures is more practical due to their flexibility as far as growth conditions and operational parameters are concerned. Non-sterile conditions would also be acceptable, thus decreasing the cost and complexity of the operation (Waligórska, 2012). Mixed cultures also use a greater variety of substrate types as the microbial community within might assist in the degradation of complex organic molecules even if they are incapable of producing hydrogen themselves (Hung *et al*., 2011). They could also use up any existing oxygen in the reactor and enhance the formation of biomass granules essentially leading to superior productivity of hydrogen (Hung *et al*., 2011). Nevertheless, if the mixed culture contains microorganisms that compete for the carbon source or consume hydrogen, such inoculum would have to undergo various physical or chemical pre-treatments like heat, acidification, alkylation, chloroform or acetylene treatment prior to hydrogen production (Akutsu *et al*., 2009; Kang *et al*., 2012; Hu and Chen, 2007; Ren *et al*., 2008; Zhu and Beland, 2006). Pure cultures of *Clostridium spp.* and *Enterobacter spp.* are commonly used in biohydrogen production; several other genus have also been used (Table 1). Mixed cultures used for biohydrogen production are commonly sourced from sewage sludge, either human or animal (Table1).

2.3.2. Substrate

First generation biofuels are made from crops associated to food supply which makes them an undesirable choice for hydrogen production (Waligórska, 2012). Second generation biofuel production can be made from plants such as sweet sorghum (*Sorghum bicolor* (L.) Moench), switch grass (*Panicum virgatum L.*) and miscanthus (*Miscanthus×giganteus* Greef et Deuter) which are known to grow in less demanding soil conditions (Alexopoulou *et al.*, 2015; Reddy *et al.*, 2005; Waligórska, 2012). Lignocellulose substrates of woody origin may also be sometimes used. Their high cellulose, lignin and hemicellulose content make them somewhat more difficult to break down, and it is during such fermentations that mixed cultures of inoculum become especially useful (Waligórska, 2012). Substrates are chosen based on their price and availability. Pure substrates such as glucose, sucrose, lactose, xylose and starch (Table 1) are commonly used in experimental studies for the purposes of experimental reproducibility. Wastes, such as cellulosic biomass, sewage sludge, chitinous waste and molasses (Table 1) are more likely to be used in industry, as they would be more cost effective.

Table 1: Inoculum involved in Hydrogen Production via Dark Fermentation

Inoculum Type	Substrate Type	Hydrogen Yield	Reference
Clostridium acetobutylicum	Glucose	2.0 mol mol^{-1} glucose	Chin *et al.,*2003
Clostridium butyricum CGS5	Xylose	0.73 mol mol^{-1} xylose	Lo *et al.*, 2008
Clostridium butyricum	Glucose	1.4- 2.3 mol mol^{-1} glucose	Kataoka *et al.*, 1997
Clostridium pasteurianum CH_4	Sucrose	2.07 mol mol^{-1} hexose	Lo *et al*., 2008
Clostridium paraputrificum M-21	Chitinous wastes	2.2 mol mol^{-1} substrate	Evvyernie *et al.*, 2001
Clostridium thermocellum 27405	Cellulosic biomass	2.3 mol mol^{-1} glucose	Levin *et al.*, 2006
Clostridium thermolacticum	Lactose	3.0 mol mol^{-1} lactose	Collet *et al.*, 2004
Enterobacter aerogenes	Starch	1.09 mol mol^{-1} starch	Fabiano and Perego, 2002
Enterobacter aerogenes E 82005	Molasses	3.5 mol mol^{-1} sugar	Tanisho and Ishiwata, 1995
Enterobacter cloacae IIT-BT 08	Sucrose	6 mol mol^{-1} sucrose	Kumar and Das, 2000
Enterobacter cloacae IIT-BT 08	Cellobiose	5.4 mol mol^{-1} cellobiose	Kumar and Das, 2000
Thermoanaerobacterium thermosaccharolyticum KU001	Glucose	2.4 mol mol^{-1} glucose	Ueno *et al.*, 2001
Thermotoga elfii	Glucose	84.9 mmol L^{-1} medium	van Niel *et al.*, 2002
Ruminococcus albus	Glucose	2.52 mol mol^{-1} glucose	Ntaikou *et al.*, 2008
Citrobacter amalonaticus Y19	Glucose	8.7 mol mol^{-1} glucose	Oh *et al.*, 2008
Ethanoligenens harbinense YUAN-3	Glucose	1.93 mol mol^{-1} glucose	Xing *et al.*, 2008
Digested sludge	Glucose	1.8 mol mol^{-1} glucose	Wang and Wan, 2008
Enterobacter aerogenes HU-101	Glucose	1.17 mol mol^{-1} glucose	Mahyudin *et al*., 1997
Digested wastewater sludge	Sucrose	6.12 mol mol^{-1} sucrose	Zhu and Béland, 2006
Rhodopseudomonas	Glucose	2.76 mol mol^{-1} glucose	Oh *et al.*, 2002
Cattle manure sludge	Glucose	1.0 mol mol^{-1} glucose	Cheong and Hansen, 2006
Enterobacter aerogenes	Molasses	1.58 mol mol^{-1} molasses	Tanisho *et al.*, 1998
Mixed mesophillic microflora	Sewage sludge	1.7 mol mol^{-1} substrate	Lin and Chang, 1999
Methanogenic granules	Glucose	1.2 mol mol^{-1} glucose	Hu and Chen, 2007
Anaerobic sludge	Dairy waste	0.0317 mmol g^{-1} COD	Mohan *et al.*, 2008

2.3.3. Hydrogen Partial Pressure (HPP)

A high hydrogen partial pressure within the reactor negatively impacts hydrogen production by influencing the metabolisms of the microorganisms toward the synthesis of reduced compounds rather than hydrogen (Waligórska, 2012). As the concentration of hydrogen increases in the liquid phase fermentation broth the hydrogenase enzyme tasked with producing hydrogen is inhibited and the thermodynamics become unfavourable (Jung *et al*., 2011; Show *et al*., 2011; Tiwari *et al*., 2006). Various strategies have been employed to control the hydrogen partial pressure in fermentations ranging from gas sparging, the use of a submerged membrane, use of a vacuum, to thermodynamic regulation (Hussy *et al*., 2003; Hussy *et al*., 2005; Kataoka *et al*., 1997; Kim *et al*., 2006; Kraemer and Bagley, 2006; Kraemer and Bagley, 2007; Kyazze *et al*., 2006; Liu *et al*., 2006; Mandal *et al*., 2006; Mizuno *et al*., 2000). The presence of nitrogen gas in the bioreactor has many benefits on the hydrogen producing reactions, as it displaces the carbon dioxide within the void spaces of the reactor, thereby inhibiting any potential acetogenic hydrogen consuming reactions (Mizuno *et al*., 2000). Unfortunately, continuous sparging also results in incomplete glucose consumption (Kim *et al*., 2006). Agitation facilitates the transfer of metabolic gases from the fermentation broth to the reactor headspace (Hawkes *et al*., 2007; Hawkes *et al*., 2002). Since this occurs repeatedly, especially during the exponential hydrogen production phase, the gas produced is swiftly transferred from the fermentation broth to the headspace and subsequently out the reactor into the analysis equipment (Hawkes *et al*., 2002). This succession of events promotes the expulsion of hydrogen gas from the reactor, thus continually reducing the hydrogen partial pressure (Hawkes *et al*., 2002).

2.3.4. Temperature

The temperatures of the reactions for hydrogen production depend on the type of microorganisms used in the inoculum. Thermophillic organisms are less susceptible to the effect of hydrogen partial pressure and contamination (Waligórska, 2012). Additionally, inoculum pre-treatment may be omitted as it has been reported that methanogens were inactive at thermophillic temperatures (Shin *et al*., 2004; Valdez-Vazquez *et al*., 2005). Temperature ranges of between 50-60°C have been used to optimise biohydrogen using thermophillic microorganisms (Jung *et al*., 2011).

Generally, thermophillic organisms have been found to yield a smaller hydrogen volume as compared to mesophillic organisms; this would make it necessary to scale up the size of the reactors, thus increasing the overall cost (Hallenbeck, 2009). Coupling thermophillic process in tandem with an exothermic process via a heat exchanger would be economically viable, since running such a process independently would be very demanding practically and economically (Hawkes *et al*., 2007). Temperatures between 33°C and 41°C have been shown to improve energetic efficiency within mesophillic organisms (Mu *et al*., 2006a,b).Varying heat treatments are also used to treat inoculum to harvest the hydrogen producing population from a mixed culture (Logan *et al*., 2002).

2.3.5. pH

The pH of the system affects the enzymes employed by the inoculum, thus in a domino effect affects the organism's metabolism, community structure and product formation (Ye *et al*., 2007). The origin of the inoculum used as well as the substrate used also plays a role in the optimum pH chosen. The suggested pH for substrates such as food waste falls between 5 and 6 (Guo *et al*., 2010). Optimal pH for animal manure and agricultural waste substrates falls within the neutral region (Guo *et al*., 2010). It may be advised that experimentation be done to determine the optimal pH of the process being studied as the composition of waste substrates as well as inoculum varies considerably. Running processes at lower pH values could also assist in decreasing the growth of methanogens, known for consuming hydrogen (Hawkes *et al*., 2002).

2.3.6. Hydraulic retention time (HRT)

During continuous process the hydraulic retention time affects the yield of biohydrogen. Short HRT promotes hydrogen producing microorganisms (Jung *et al*., 2011), times between 8-12 hours was found to be optimum for liquid media. The time increases depending on the structural complexity and viscosity of the substrate used (Hawkes *et al*., 2002). Short HRT also inhibits the growth of methanogenic microorganisms as they require longer times to grow which is an added advantage for optimizing biohydrogen production (Jung *et al*., 2011).

2.3.7. Volatile fatty acids

The presence of various compounds within the effluent of a fermentation process provides useful indicators of the various metabolic pathways that occurred within. Metabolic pathways favouring the production of acetic acid are most favourable for the production of hydrogen gas (Hawkes *et al*., 2002; Hawkes *et al*., 2007; Kraemer and Bagley, 2007). This pathway has the highest molar hydrogen yield (Kraemer and Bagley, 2007; Hawkes *et al*., 2007). The production of butyrate is less preferred (Kraemer and Bagley, 2007), as more hydrogen atoms are used in the molecule and therefore less hydrogen gas is produced. However, the production of butyrate or butyric acid is still preferred over propionate and lactate as these by-products indicate that no hydrogen gas was produced (Hawkes *et al*., 2002). The production of alcohols are least preferred as these molecules also contain more hydrogen and would therefore be present in pathways yielding less hydrogen gas (Hawkes *et al*., 2002). Additionally, alcohols such as ethanol inhibit growth and hydrogen gas production rates (Hawkes *et al*., 2002). Reduced fermentation products, such as ethanol, butanol, and lactate are a representation of hydrogen that has not been liberated as a gas but has been bound in the molecule, these products should be avoided (Hawkes *et al*., 2002). It has been suggested that volatile fatty acids are the preferred by-product as metabolic pathways that yield them concurrently yield hydrogen gas (Hawkes *et al*., 2002; Lay, 2000). Lamed *et al*. (1988) determined that mixing decreases the ethanol to acetate ratio that occurs during a fermentation process, this is an indication that agitation would therefore improve hydrogen production as acetate and hydrogen are produced simultaneously and ethanol is an inhibitor of this process. The production of alcohols occurs immediately after the peak phase of hydrogen production and volatile fatty acids (Lay, 2000; Lay *et al*., 1999; Lay, 2001), in the late growth phase (Lay, 2000). This was unanimous for a pure Clostridia culture and mixed cultures obtained from municipal solid waste and heat-treated sludge (Lay, 2000; Lay *et al*., 1999; Lay, 2001).

2.4. Biohydrogen yield

A theoretical hydrogen yield of 4 mol H_2 mol^{-1} hexose is possible, if acetic acid is the only by product (Hawkes *et al*., 2007; Kraemer and Bagley, 2007). In laboratory experiments carbohydrate substrates are also utilized for cellular proliferation and

formation of alternative by-products (Hawkes *et al*., 2007), consequently decreasing product formation. Additionally, mixed cultures contain an assortment of microorganisms and their respective metabolic pathways may sometimes vary. This would cause the molar hydrogen yield to differ depending on which microorganisms are selected via varying pre-treatment techniques (Yang *et al*., 2019). Molar yield may be improved by channelling the effluent (containing by-products) of stage one fermentation into stage two fermentation, as the substrate (Hawkes *et al*., 2007).

Agitation of the reaction medium within the bioreactor improves hydrogen yield (Hawkes *et al*., 2002; Kraemer and Bagley, 2007; Lamed *et al*., 1988; Lay, 2000). Photo-fermentation or utilization of efficient microbial fuel cells was proposed to improve overall hydrogen yield mol^{-1} hexose in a secondary fermentation (Hawkes *et al*., 2007; Hawkes *et al*., 2002). A second stage of fermentation yielding other useful and high energy end products, like fertilizer rich in nitrogen/phosphorus and methane, may be considered to improve the overall economics and practicality of the process (Hawkes *et al*., 2002).

2.5. Bioreactor configurations used for biohydrogen production

The bioreactor type used impacts the growth of the microorganism and in essence the yield and quality of the product formed. Therefore, the selected reactor configuration needs to meet the requirements of the microorganism and yet show maximum product yield as well. Among the various parameters that determine the hydrogen production efficiency, the reactor design plays a key role in optimizing hydrogen production yield (Venkata mohan *et al*., 2007b; Cresson *et al*., 2008). Bioreactor design also has a major influence on the interaction between microorganism and substrate, the amounts of microorganisms present and the hydrodynamic behaviour of the solutions within the reactor (Venkata mohan *et al*., 2007b; Cresson *et al*., 2008). Essential requirements such as temperature, gas exposure and pH play a role in ensuring harmonious internal environment within the reactor.

2.5.1. Continuous stirred tank reactor (CSTR)

The continuous stirred tank reactor (Figure 1) vessels are designed to ensure efficient mixing of the substrate within the reactor; preventing settling of nutrients and promoting efficient mass transfer (Ntaikou *et al*., 2010). This reactor type is commonly employed in conjunction with a continuous flow mode (Yu *et al*., 2003). Challenges

encountered herein depend on the hydraulic retention time (HRT), if it is too short wash out of biomass may occur, if it is too long promotion of methanogenic hydrogen consuming bacteria may be promoted (Waligórska, 2012). Even though this reactor type has the most efficient mixing capabilities, the impeller system used can result in cell shearing and lysis, therefore leading to a lower biomass concentration (Zhong *et al*., 1994), and by extension a decreased hydrogen yield. Studies using CSTR with 10g L^{-1} glucose at a pH of 5.5 and temperature of 37°C had a biohydrogen yield of 1.81ml H_2 mol^{-1} (Show *et al*., 2011) at a production rate of 3.20 L h^{-1} L $^{-1}$ (Show *et al*., 2007; Zang *et al*., 2007). Other studies using sucrose had a hydrogen production rate of up to 15 L h^{-1} L^{-1} (Wu *et al*., 2006).

Figure 1 shows a diagrammatic representation of a continuous stirred tank reactor. The agitation system consists of the motor, blender/impeller and the baffles.

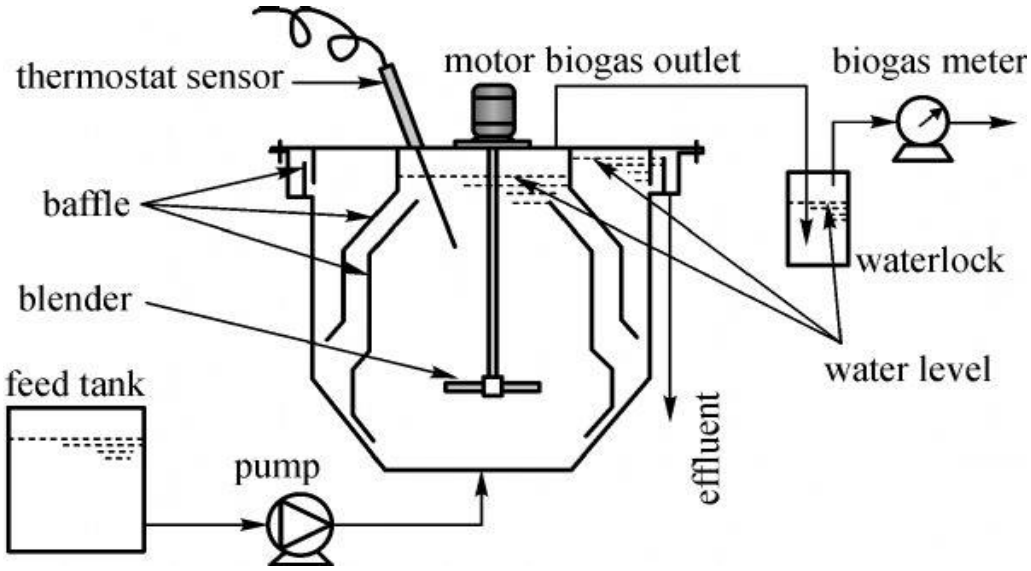

Figure 1: Schematic diagram of a continuous stirred tank reactor (Zhu *et al*., 2013).

2.5.2. The membrane bioreactor (MBR)

The membrane bioreactor consists of a membrane upon which the inoculum is immobilized (Figure 2); this benefits product formation as it prevents biomass washout because the cells are fixed to the reactor (Show *et al*., 2008). The aforementioned system can be inefficient if the microorganisms produce extra polymeric substances (EPS) which result in fouling of the membrane. This is one of the key reasons that the reactor type is not used industrially (Show *et al*., 2008). Due to the biomass being fixed onto the membrane, the emanating effluent would thus contain greatly reduced levels

of the microbial population. Additionally, the reactors can be smaller in size since this reactor type is more efficient at consuming organic substrates and is able to produce hydrogen at a faster rate (Oh *et al*., 2004). A disadvantage of this system is that a long solids retention time (SRT) can result in a swift increase in biomass and a decrease in the rate and efficiency of the hydrogen production (Lee *et al*., 2011). Studies using a membrane bioreactor have yielded a hydrogen production rate of between 0.50 to 0.64 L h^{-1} L^{-1} using a HRT of 3.3 hours (Oh *et al*., 2004).

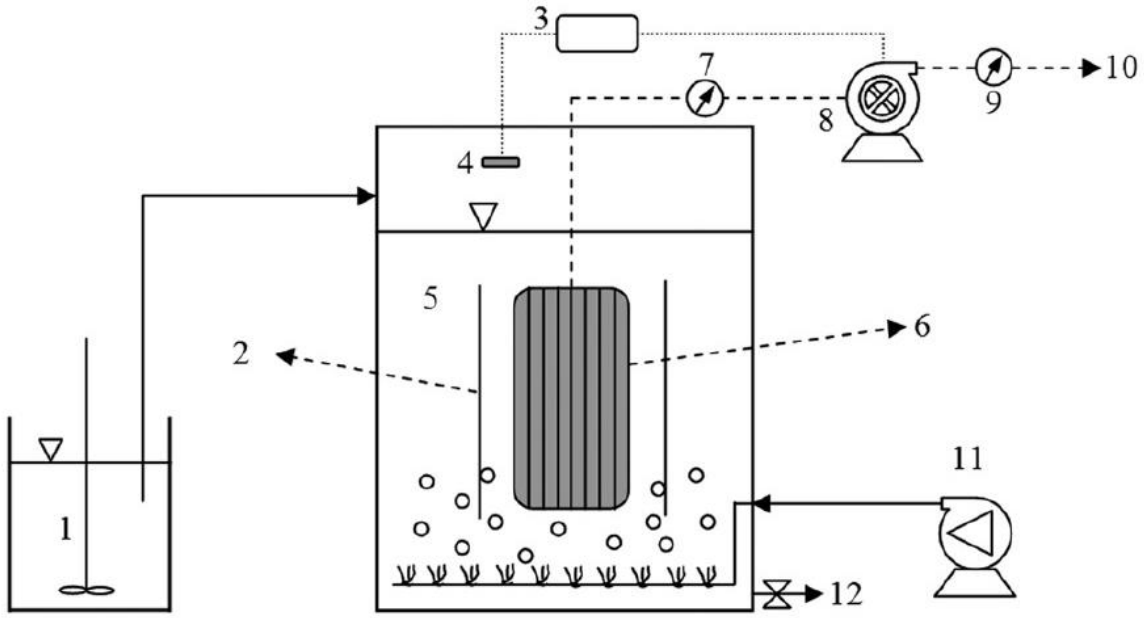

Figure 2: Schematic diagram of a flat sheet membrane bioreactor (Fazeli *et al*., 2012)

Figure 2 shows a diagrammatic representation of a membrane bioreactor. Note should be taken of component 6, the membrane of the module, which was the unique feature of this bioreactor configuration.1- Reservoir, 2- baffle plate, 3- PLC, 4- sensor, 5- bioreactor, 6- membrane module, 7- vacuum gauge, 8- suction pump, 9- flow meter, 10- permeate, 11- blower and 12- waste sludge.

2.5.3. Packed bed reactors (PBR)

Packed bed reactors are configured to allow entry of substrate from the bottom and exit from the top (Figure 3), the cellular biomass is generally immobilized either on biofilms or granules captured on packed media (Kothari *et al*., 2012). The support media generally consist of either granular activated carbon (GAC), sponge,

polyethylene-octane elastomer, ceramic ball, alginate gel or expanded clay (Show *et al*., 2011). The key downfall of this reactor type is that the substrate supply is less efficient compared to the other reactor types previously described. The lack of efficient substrate results in a reduced substrate usage leading to a decreased hydrogen yield (Show *et al*., 2011); which may be attributed to the system lacking proficient mixing, so as to unify the substrate concentration throughout the reactor equally. The supply of substrate to the reaction vessel could be enhanced by recycling the substrate back into the reactor. Studies have shown that this technique has further enhanced the hydrogen production yield and efficiency (Kumar and Das, 2001). Show *et al*. (2011) indicated that using this reactor configuration at an HRT of 1.08 hours a hydrogen production rate of 1.60L h^{-1} L^{-1} was achieved. Furthermore, it was also established that the hydrogen production efficiency improved when the substrate was recycled through the reactor repeatedly.

Figure 3 shows that the inside of the packed reactor consists of immovable packaging, the motion of the substrate moves through the packaging to allow the microbial cells trapped within access to a homogenous flow of nutrients. Any product formed, exits at the fluid out port. Alternatively, a special gas exit port will need to be inserted above the substrate level for a gas product.

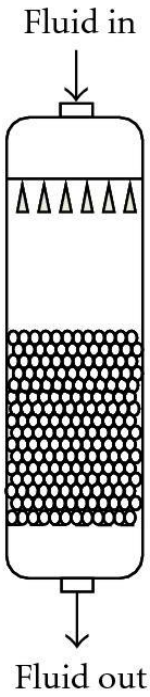

Figure 3: Schematic diagram of a packed bed reactor (Fernandes, 2010).

2.5.4. Fluidized bed reactors (FBR)

Fluidized bed reactor systems (Figure 4) require large amounts of gas or liquid to be pumped into the reactor to facilitate the mixing of the contents (Waligórska, 2012). The biomass generally gets immobilized onto materials similar to those used in the packed bed reactor configuration (Waligórska, 2012). However, these materials are smaller in size so as to allow ready fluidization and to increase the surface area of the immobilization particles so that the microorganisms entrapped therein would be exposed to a greater degree, thus promoting a better mass transfer and higher hydrogen yield (Barros *et al*., 2010). This reactor configuration is also well known for its highly proficient mixing abilities. Especially high hydrogen yields have been noted from cells immobilized on granular activated carbon (GAC) (Zang *et al*., 2007) compared to other materials used for immobilization. Lin *et al*. (2009) found that a rate of 1.821 L h^{-1} L^{-1} hydrogen was produced from a sucrose substrate with a yield of 4.26 mol H_2 mol^{-1} sucrose with a HRT of 2 to 6 hours at steady state.

Figure 4 indicates that the immobilization matrix within the bioreactor moves with the momentum of the surrounding media thus the agitation affects both the immobilized cells and the media within the bioreactor.

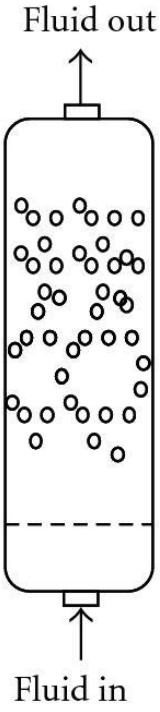

Figure 4: Schematic diagram of a fluidized bed reactor (Fernandes, 2010).

2.5.5. Up-flow anaerobic sludge blanket (UASB) reactor

The up-flow anaerobic sludge blanket reactor (Figure 5) employs a system that allows the entry of the effluent in the form of substrate to enter the reactor from the bottom and the exit of the treated materials form the top (Seghezzo *et al*., 1998) .This reactor type had most commonly been used in conjugation with methane production (Waligórska, 2012). The most pronounced disadvantage of this system is the time factor involved prior to the start of the reaction processes (Wang *et al*., 2007). Some studies indicate that the reactor needs up to 5 months to form the blanket of immobilized cells in order for the reactor to function optimally (Wang *et al*., 2007). This process can however be sped up by encouraging granulation within a CSTR and thereafter transferring the materials to a USAB for the actual digestion (Waligórska, 2012). In industrial processes, time is essential and more often than not waiting 5 months for a reactor to stabilise is far too long especially if there are more timeous and less tedious means of achieving the same objective (Wang *et al*., 2007). A hydrogen production rate of 0.25 L h^{-1} L^{-1} was reported whilst using a UASB reactor fed with a sucrose substrate at a HRT of between 8 to 20 hours the hydrogen yield was 1.5 mol H_2 mol^{-1} sucrose (Chang and Lin, 2004).

Figure 5 shows effluent entering form the bottom of the reactor, being treated by the sludge granules formed over time naturally, treated water exists from the top of the reactor.

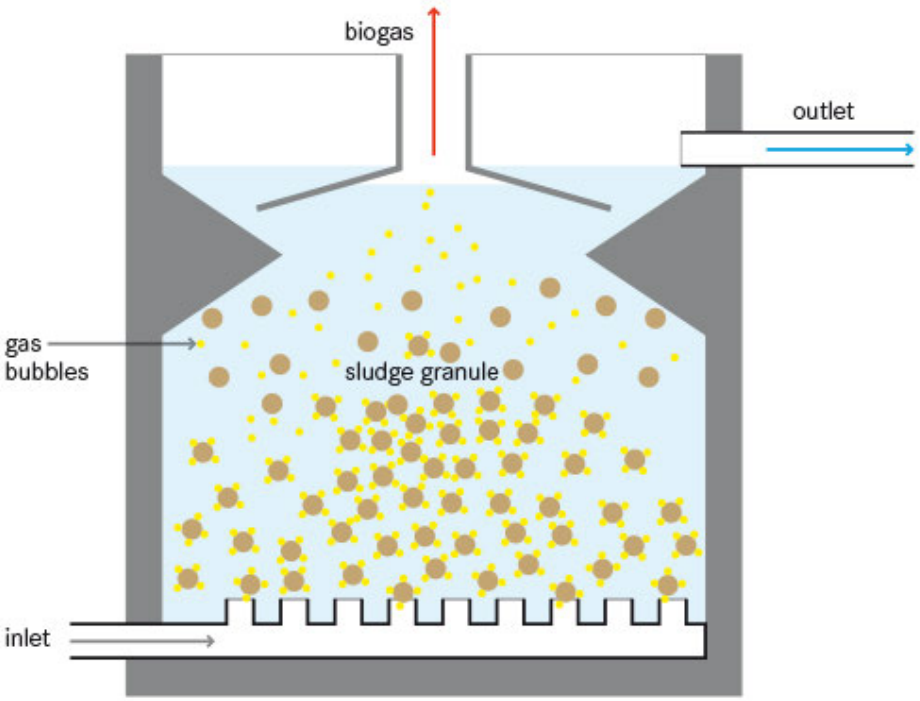

Figure 5: Up-flow anaerobic sludge blanket (UASB) reactor (Tilley *et al*., 2014).

2.5.6. Temporary immersion reactor

Temporary immersion bioreactors, commonly referred to as temporary immersion systems (TIS), are reactors that allow the inoculating organisms' exposure to air and a liquid substrate at sequential intervals (Georgiev *et al*., 2014). Various similar designs exist to achieve this objective (Watt, 2012). The earliest design consisted of using two separate vessels connected via tubing (Figure 6). One vessel contained the immobilized growth organism and the other a liquid substrate (Figure 6). The operation system in this early twin-flask design (Figure 6) was pneumatic and individually controlled by clocks that initiated the transfer of the substrate from the holding flask to immerse the growing cells and back to the holding flask again. Some other TIS designs include the Ebb-and-flow, RITA (recipient for automated temporary immersion), rotating drum and wave bioreactor, these configurations utilize an array of agitation techniques ranging between pneumatic, gravity, hydraulic and mechanical (Georgiev *et al*., 2014).TISs are commonly used for tissue culture work, particularly plant culturing (Etienne and Berthouly, 2002; Robert *et al.*, 2006). Their purpose was to assist in reducing the plant exposure to toxic quantities of water and increase the oxygen availability, to prevent hyperhydricity and asphyxia (Debnath, 2011).

Unimpeded contact between the air and plant material reduces the resistance between interface boundary layers (gas-liquid and liquid-solid interfaces) (Curtis and Tuerk, 2008). Therefore atmospheric oxygen is able to enter the culture cells with greater ease than if the culture cells were within a submersion fluid (Georgiev *et al*., 2014). The inversion of this characteristic is what holds hypothetical prospect for hydrogen production. If the direct contact of air to inoculating cells allows them unhindered access to gaseous nutrients present in the air, relatively they would be able to release gaseous product into the air with the same ease, as opposed to being exclusively submerged. Furthermore, the motion of the submersion and drainage causes enhanced turbulence, increased exposure between inoculum and substrate and thorough homogenisation of the bioreactors macro and micro environments. The agitation system also utilizes gravity to aid its function in some designs (Robert *et al*., 2006), thereby reducing energy input requirements. The reactors dynamics, internal constituents, preparatory procedures and frequency of submersion would have to be modified to cater to the specific requirements of biogas/hydrogen production.

Figure 6 shows the a stepwise process of the operation of the twin-flask system, A- growth organisms experiencing exposure to air, B- liquid substrate being transferred to immobilized growth organism, C- growth organism experiencing an immersion period, D- liquid substrate being transferred back to holding flask.

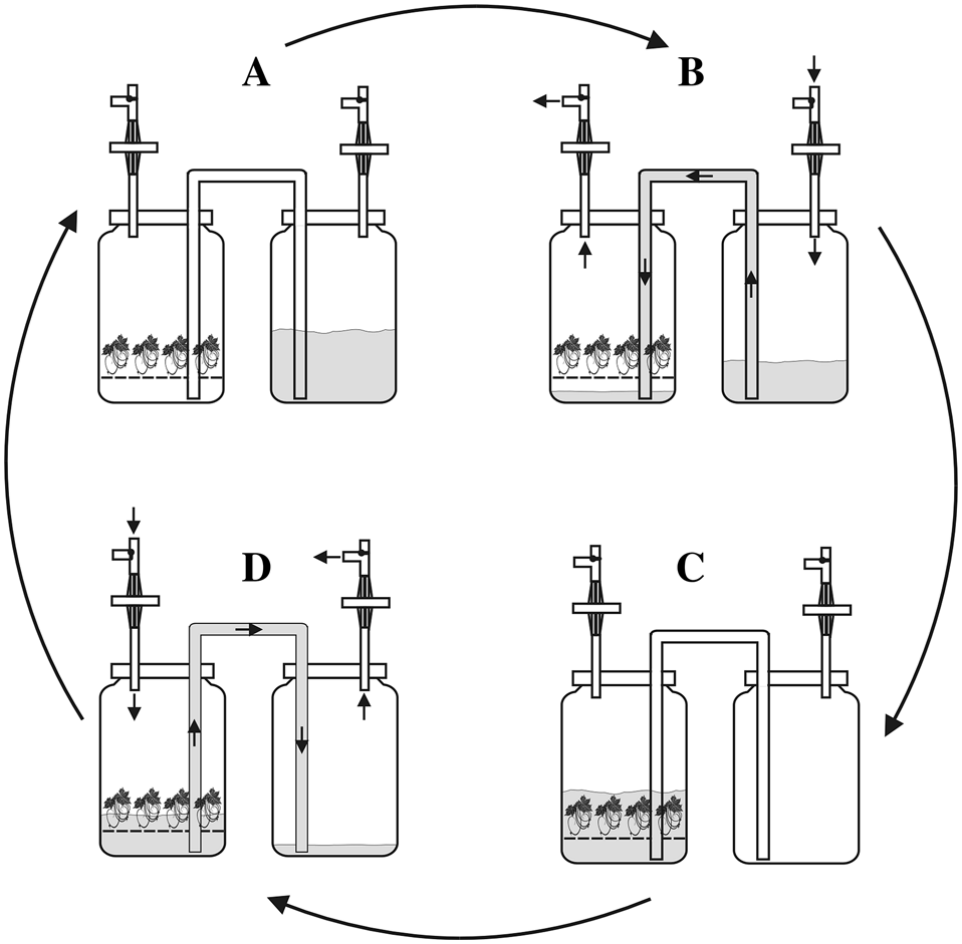

Figure 6: Twin-flask temporary immersion system (Georgiev *et al*., 2014)

2.6. Process mode

There are various opinions expressed in literature as to which mode of fermentation would be most beneficial for optimal hydrogen production. Some researchers are of the opinion that a batch mode is appropriate for research purposes and give a satisfactory indication of the growth capabilities of the inoculum in question (Waligórska, 2012). Continuous flow reactors are appropriate for industrial purposes due to the large volumes of waste that require treatment and the large volume of product that are necessary (Ntaikou *et al*., 2010). In contrast to this view some researches are of the opinion that the batch mode produces inefficient and stunted data (Show *et al*., 2008). They suggest that it is incapable of providing a good indication of the best hydrogen production rates, and that a semi-continuous mode would be a better comparison to an industrial production (Show *et al*., 2008). Processes involving a form of semi-continuous mode with a mixed population of

microorganisms from activated sludge were undertaken by Cohen *et al*. (1985). A series of timeous feed interruptions were enacted on the process to investigate its effect on the metabolic products formed in the reactor. The team theorised, based on the results of their investigation that the metabolisms of these anaerobic organisms shifted from spore forming butyrate/H_2 producers to non-spore forming propionate producers. This change occurred despite the range of interruption patterns utilized in the investigation. This is an indication that the semi-continuous mode of operation might be problematic when a mixed consortia of microorganisms is employed as the inoculum source. Batch mode process are considered more suitable for initial optimization studies (Hallenbeck and Ghosh, 2009) as it is easier to determine the degree of optimization and whether said optimisation is as a result of the novel reactor configuration rather than a variation in the operating conditions of a bioreactor vessel. As the scale of the operation increases the mode of the process would have to be run as a continuous feed (Hallenbeck and Ghosh, 2009) to cater to the large volumes of wastewater. Additionally, production of a reasonable quantity of hydrogen gas is required to allow the production plant independent operation and contribute to the local power grid.

2.7. Some considerations for an effective bioreactor for biogas production

The process employed to produce hydrogen is influenced by various chemical and physical parameters. Suitable mixing determines the level of exposure the inoculating organisms has to the heterogeneous substrate, the state of the hydrogen partial pressure, the volatile fatty acids produced, hydraulic retention time and the level of homogeneity in the pH and temperature. Immobilization of microorganisms liberates growth rate from hydraulic retention time, which prevents inoculum washout. Reactors that are designed to immobilization often lack suitable agitation for DFHP and rely on air or natural substrate flow to expose the inoculum to the substrate. Ineffective agitation with heterogeneous substrates may also lead to elevated hydrogen concentrations in the liquid phase. The HRT of the process would also have to be increased to allow adequate substrate degradation. Immobilized microorganisms require more agitation than suspended organisms to be suitably exposed to the substrate. The degree of agitation cannot however be so vigorous so as to disintegrate

the immobilised structures or to dislodge the microbial cells from the immobilization matrix. Various bioreactor designs commonly used in experiments for fermentative hydrogen production can either allow suitable agitation at the cost of compromised immobilization. Alternatively they immobilize cells with insufficient agitation, ineffective mass transfer and an accumulation of hydrogen gas in the liquid phase. Based on these observations, a novel bioreactor configuration to encapsulate immobilization and effective non-invasive agitation is necessary. A novel bioreactor design has the potential to enhance biogas production. The combination of immobilization, efficient agitation, reduced hydrogen partial pressure, superior exposure of inoculum to substrate and continued thorough homogenisation of the fermentation broth may improve the yield and rate of hydrogen production substantially.

2.8. Conclusion

A variety of chemical parameters and bioreactor configurations were reviewed. In conclusion immobilization of inoculum has many benefits to improve biohydrogen production and short hydraulic retention times have a positive impact on overall yield. The continuous stirred tank reactor maintains highly efficient agitation but may require modifications to conduct processes with effective immobilization. Other reactor configurations with the capability to maintain immobilized cells efficiently, like the packed bed reactor and anaerobic sludge blanket reactor may lack an adequate mixing ability for high throughput dark fermentative hydrogen production. A reactor configuration that aims to merge effective immobilization and efficient agitation is necessary. Such a design would be a highly commendable dynamic immobilized system. Furthermore, this reactor would be suitable for other industrial processes that would benefit from immobilization with substantial and effective agitation.

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Chapter 3

Design, implementation and assessment of a novel bioreactor for fermentative biohydrogen process development

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This chapter has been published with the title: Design, implementation and assessment of a novel bioreactor for fermentative biohydrogen process development in the International Journal of Hydrogen Energy (2016; 41: 10136-10144)

The article is presented in the following pages.

INTERNATIONAL JOURNAL OF HYDROGEN ENERGY 41 (2016) 10136–10144

Available online at www.sciencedirect.com

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journal homepage: www.elsevier.com/locate/he

Design, implementation and assessment of a novel bioreactor for fermentative biohydrogen process development

*M.B. Hassan Khan, E.B. Gueguim Kana**

Discipline of Microbiology, School of Life Sciences, University of KwaZulu-Natal, Private Bag XO1, Scottsville, 3209 Pietermaritzburg, South Africa

ARTICLE INFO

Article history:
Received 30 October 2015
Received in revised form
4 April 2016
Accepted 28 April 2016
Available online 18 May 2016

Keywords:
Novel biogas bioreactor
Dark fermentation
Biohydrogen
3-D printing
Cell immobilization

ABSTRACT

Hydrogen production using dark fermentation is challenged by a low yield. Factors affecting the yield include the reactor configuration, the microbial community structure and other physico-chemical parameters. This paper reports the implementation and assessment of a novel reactor type. The vessel was made up of a 2-L glass with five ports. Three 3D-printed porous cartridges supported the immobilized microbial cells and served as baffles. Mixing was achieved by rocking the vessel at 180°. All the control set points could be varied, offering the possibility of assessing various control regimes. For biohydrogen production, the implemented reactor showed 58 and 60% reduction in lag time for immobilized and suspended culture respectively, and 35 and 12% increase in peak hydrogen fraction for the immobilized and suspended cells respectively, compared to the flask reactor. These findings highlight the capabilities of this reactor configuration to enhance the productivity and yield of biohydrogen.

Introduction

The majority of current energy sources originate from fossil fuels and concomitant compounds. These sources, as convenient as they are, also contribute to the steady and climaxing degeneration of the earth's atmosphere and most importantly the ozone layer [1]. It is estimated that over 87% of the world's energy consumption is derived from fossil fuels [2]. The cataclysmic natural disasters occurring globally could be attributed to the swift environmental changes as a result of the continued use of fossil fuels and their derivatives. The energy crisis could be elevated if renewable sources are not explored.

Hydrogen is a suitable alternative due to its fervent combustibility and high energy yield of 122 kJ/g [4] whether explosively in heat engines or silently in fuel cells [3]. Hydrogen energy yield surpasses that displayed by equivalent amounts of hydrocarbon fuels by yielding 2.75 times more energy [4]. Upon the combustion of hydrogen the only by-product is water which does not require extravagant and costly means of disposal or storage, moreover it has no adverse side effects on the planet [3]. Hydrogen could be produced in light dependent reactions like the biophotolysis of water using cyanobacteria and algae or photodecomposition of organic substrates by photosynthetic bacteria [3]. Another widely researched field proficient in hydrogen generation involves light independent or dark fermentation of organic substrates facilitated anaerobically [3]. Fermentative hydrogen production has boundless potential as its production is based on the use of waste as substrates [5]. This method not only ensures the efficient production of a clean

* *Corresponding author*. Tel.: +27 332605527.
E-mail address: kanag@ukzn.ac.za (E.B.G. Kana).
http://dx.doi.org/10.1016/j.ijhydene.2016.04.208

renewable energy source but also degrades waste materials that would have been dumped in landfill sites [1]. The use of hydrogen as an energy source rates more preferable as opposed to the use of nuclear energy and its associated repercussions. The containment and disposal of nuclear energy sources has led to a multitude of disastrous circumstances and had extraordinarily high cost indications [6].

However, the yield of biohydrogen needs to be improved in order to make its production and industrialization economically feasible. Biohydrogen yield is affected by substrate type, microbial community structure and bioreactor configuration amongst other physico-chemical operational parameters [2]. Existing literature stresses on the importance cells immobilization on maintaining a microbial community structure favourable for biohydrogen production [7]. Laboratory studies are fundamental and necessary at various stages of the process development such as screening, modelling and optimization, process scale up and process scale down. Most investigations on fermentative hydrogen production are carried out using shake flask reactors in shaking waterbaths or rotary shakers [8–10]. Shake flask systems are however plagued by a series of imperfections which make them unsuitable for high throughput accurate data. These shortcomings include the lack of pH control, poor gas and mass transfer rooted on inefficient mixing regimes and considerable evaporation of the total reaction mixture [11]. It has been established that current bioreactor models used for biohydrogen research and production either lack efficiency in terms of agitation or have vigorous mixing systems. Impairing the integrity of the microbial cells involved in fermentation and are incapable of maintaining microbial biomass during continuous processes [3,12]. Both of which result in inferior hydrogen production yields [12]. During industrial continuous processes, short hydraulic retention times promote hydrogen production and biomass washout occurs rapidly in the Continuous Stirred Tank Reactor (CSTR) diminishing hydrogen production [12]. This has led to the exploration of other reactor configurations, like the up-flow anaerobic sludge blanket (UASBR) reactors, anaerobic membrane bioreactors and immobilized (fluidized bed) bioreactors with improved hydrogen producing potentials [13,14]. These configurations lack efficient mass transfer capabilities. Given the importance of laboratory scale studies, a more suitable reactor designed for biofuel studies addressing the above mentioned attributes will provide a more valuable process knowledge. The aim of this study was to design and implement a novel bioreactor configuration for biohydrogen research that provides adequate mixing for heat and mass transfer while maintaining the microbial community structure. The novel reactor was comparatively assessed with flask culture system on hydrogen production from synthetic wastewaters using suspended and immobilized microbial cells.

Materials and methods

Bioreactor design and implementation

The reactor vessel

The reactor vessel was a glass cylinder with a base diameter to height ratio of 1.04:1. It incorporated five ports for various sensors and actuators (pH, temperature sensors, heating fingers, gas outlet and additional ports) with dimensions ranging from 4 to 18 mm diameter (Fig. 1A). A wide opening of 135 mm diameter was introduced at one end of the cylinder to ease the insertion of internal structures of the reactor (Fig. 1A).

The reactor lid and internal components

The lid was designed to ensure airtightness by maintaining a wide rim around the circumference (Fig. 1B). The inside of lid had a groove around its circumference for a rubber seal (Fig. 1B), the internal part of the lid (Fig. 1B) was designed to house the inner plate (Fig. 1C) which held the cartridges at 90° to each other. Six U-Clamps were used to hold the lid to the vessel by clamping them together when tightened (Fig. 1D).

Three perforated rectangular cartridge structures (152mm × 34mm × 20 mm) were designed to support the immobilized cells and served as baffles for mixing (Fig. 1G). The pore sizes were 3 mm in diameter. These cartridges had a sliding upper aperture for easy loading of immobilized matrixes such as alginate beads (Fig. 1G). The cartridges were radially aligned within the bioreactor according to the indentations in the inner plate (Fig. 1C). The inner plate was wedged inside the lid, and cartridges installed into the rectangular holes of the inner plate at 90° to each other (Fig. 1F). This conformation was selected to allow maximum exposure between the immobilized microorganisms housed inside the cartridges and the liquid media within the bioreactor vessel. The clamps were placed around the circumference of the overflows 60° apart (Fig. 1F).

Bioreactor support stand

A stainless stand (Fig. 1E) was designed to support the bioreactor vessel and the mixing motor. The side panels of the stand were cut to enhance the visual assessment of the bioreactor's content while in motion during the fermentation process. The height and angle of protruding arms from the base were made adjustable to optimise the smooth rotation and to provide compatibility with other vessels of various sizes (Fig. 1E). Rubber lined bearings with an outer diameter of 20 mm were mounted at the apex of each arm to reduce friction during the agitation of the vessel (Fig. 1E).

Bioreactor implementation

A 2 L SCHOTT bottle was selected as the reactor vessel based on its ability to withstand temperatures above 120 °C and pressures of up to 150psa, with a wall thickness of 5 mm. It was modified to include various ports for sensors and actuators such as pH, temperature probe, heating fingers, gas outlet and additional ports (Fig. 2A). The glass vessel was modified as specified in the design (Fig. 1A).

The bioreactor lid (Fig. 2B) was made from Polyamide (Nylon), a material with mechanical, thermal and chemical resistant properties. The clamps (Fig. 2C) were made from Vesconite, a material which is highly resilient of abrasive wear. The screws on the surface of the clamps (Fig. 2C) tighten the lid to the glassware to prevent the exit and entry of liquid or gas.

The internal components (Fig. 2D and E) were 3-D printed on a REPRAP 3D printing machine (REPRAP, United Kingdom), using 80 m of Polylactic acid (PLA) filament. The hollow perforated cartridge (Fig. 2E) was designed to supply a

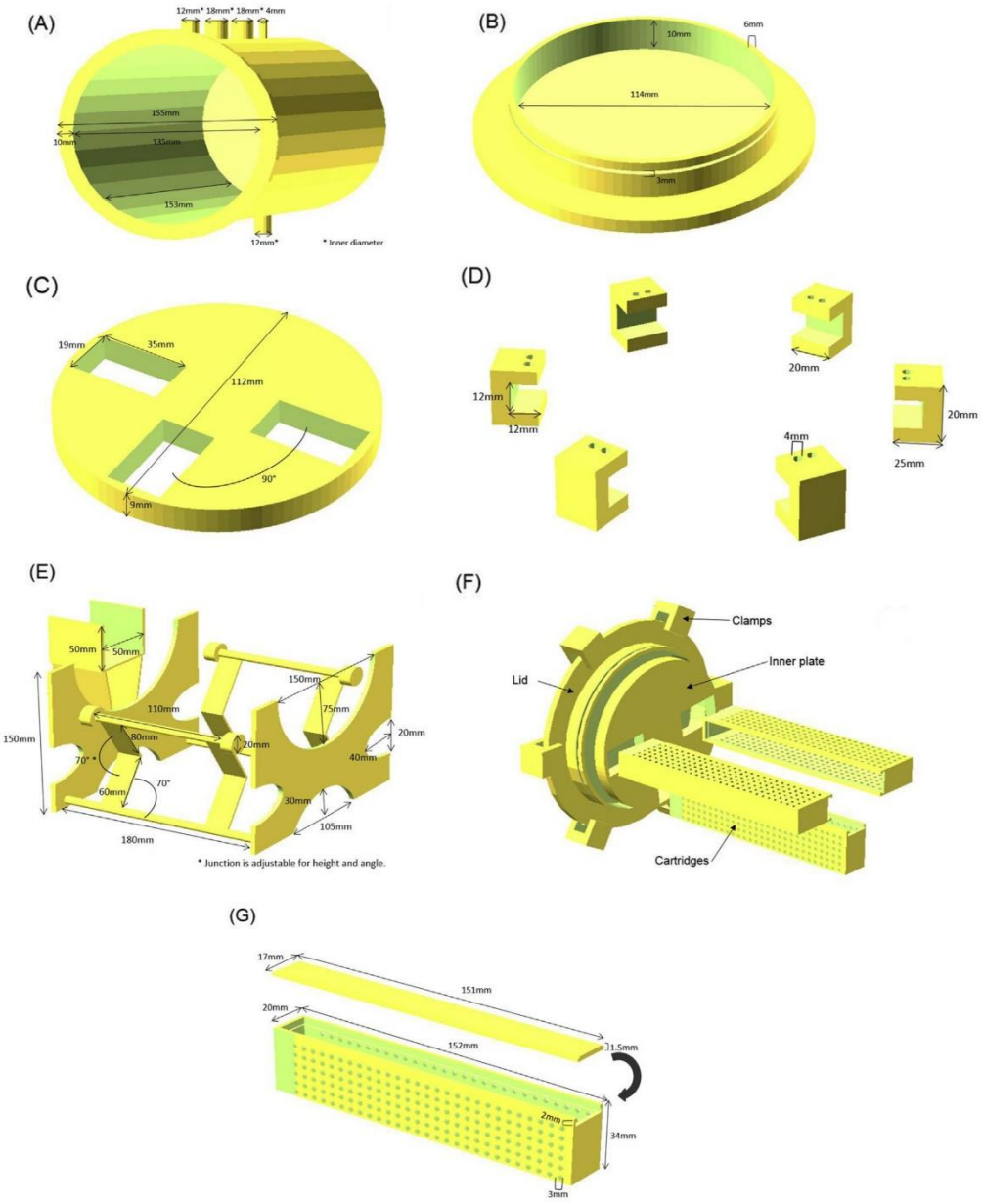

Fig. 1 – Bioreactor designed structures: (A) external vessel (B) lid internal surfaces; (C) cartridge support; (D) lid clamps; (E) bioreactor supporting stand; (F) assembled cartridges with supporting material; (G) single hollow perforated cartridge.

supporting enclosure for the immobilized microorganisms. The cartridge structure (Fig. 2E) provided an enhanced exposure of microorganisms to the substrate as a result of the perforations and the rocking motion of the bioreactor whilst being held in place by the inner plate (Fig. 2D).

The bioreactor stand (Fig. 2F) was made from stainless steel material for its ability to withstand high temperatures and chemical corrosion based on the design (Fig. 1E). The bearings and central bar linking the arms were lined with a buffering tape to reduce friction during agitation (Fig. 2F). A protrude aluminium housing was attached to the outer surface of the lid for the insertion of the motor shaft (Fig. 2G).

Bioreactor operation

Agitation/mixing

A stepper motor (Nema 17) connected to the vessel lid was programmed to rock continuously at an angle of 180° from side to

Fig. 2 – Implemented structures of the bioreactor: (A) reactor glass vessel; (B) lid internal surface; (C) vesconite clamps; (D) 3D-printed cartridge support structure; (E) 3D-printed cartridges; (F) stainless steel support stand; (G) assembled bioreactor.

side (Fig. 2G). The baffle-like cartridge structure (Fig. 2E) was filled with immobilized microbial cells (Section Assessment using Immobilised cells). This structure was designed to enhance the mixing efficiency by increasing turbulence and promoting a greater degree of exposure between the culture medium and the immobilized cells. This rocking motion allowed for a gentle and very effective mixing of the reactor contents.

Temperature control

A feedback temperature control loop was implemented using a thermistor sensor and a heating blanket actuator.

Process monitoring

A Liquid Cristal Display showed the reactor temperature in real-time and other process information such as the mixing rate and the time course of the fermentation process (Fig. 2G).

Heat transfer efficiency

Heat transfer efficiency was assessed by the time taken to raise a 1000 ml fermentation media with microbial cells by 1 °C.

Mass transfer efficiency

The mass transfer efficiency was assessed according to the modified method of Stoker [15], the reactor was filled with distilled water and the pH continuously monitored every 3 s. Once the pH of the distilled water had stabilized on 6 consecutive readings, 1 M Hydrochloric acid was added and the pH monitored until the pH of the reactor re-stabilized. The process was repeated 3 times with 1 M Hydrochloric acid and 1 M Sodium hydroxide alternately. The average time required for pH to stabilize was calculated.

Fermentation

The bioreactor was comparatively assessed with standard flask fermentation for hydrogen production using synthetic wastewater with immobilized and suspended inoculum in batch mode.

Assessment using immobilised cells

Anaerobic sludge obtained from Darville wastewater treatment facility in Pietermaritzburg, South Africa, was used in this study. A pre-treatment step consisting of maintaining the sludge at pH 8 at 25 °C for 24 h followed by heating at 121 °C for 10 min was carried out to deactivate the hydrogen consuming bacteria. Sodium alginate (Sigma) was mixed with the pre-treated anaerobic sludge to make a final 3% alginate solution containing a 50:50 alginate to sludge ratio, 0.2% granular activated carbon was added to increase bead porosity [16]. The

alginate-sludge mixture was then transferred with a peristaltic pump (Watson-marlow 503U) into an equal volume of a 0.2 M $CaCl_2 \cdot 2H_2O$ solution which caused the alginate to cross-link into small beads of 3 mm in diameter. Synthetic wastewater was prepared containing 1.5 g/L KH_2PO_4(Merck), 2 g/L $(NH_4)_2SO_4$ (ACE), 0.157 g/L $FeCl_2 \cdot 4H_2O$ (Merck), 0.1 g/L $CaCl_2 \cdot 2H_2O$ (Merck), 0.085 g/L $MnCl_2 \cdot 4H_2O$ (Merck), 0.178 g/L $ZnSO_4 \cdot 7H_2O$ (Merck), 0.072 g/L $Na_2MoO_4 \cdot 2H_2O$ (Merck) and 10 g/L $C_6H_{12}O_6$ (Glucose Anhydrous) (Merck) and pH adjusted to 6.8.

The bioreactor cartridges were filled with 173 g of beads with immobilized cells (10% inoculum). One litre of synthetic wastewater was fed to the reactor vessel and the reactor was flushed with nitrogen gas for 4 min. The temperature setpoint and rocking angle were set at 35 °C and 180° respectively. The control fermentation batch was run in parallel in a flask reactor within a shaking waterbath (Grant, England) at 35 °C and agitation of 300 rpm for 48 h. Biogas was collected and quantified at timeous intervals.

Assessment using suspended cells

An additional assessment of the reactor was carried out using suspended cells culture. The inoculum was pre-treated as described above but not immobilized. The bioreactor was assembled with empty cartridges placed radially inside the vessel to serve as baffles. One litre of synthetic wastewater was mixed with 150 ml pre-treated activated sludge (15% inoculum) and the pH was adjusted to 6.8., The resulting broth solution was then fed into the bioreactor and flushed with nitrogen gas for 4 min. The rocking angle and temperature were set at 180° and 35 °C and respectively. A control vessel was set up in a flask reactor and incubated in a shaking water bath at 35 °C and agitation of 300 rpm for 48 h.

Analytical procedures for both immobilized and suspended cell systems

The biogas generated was collected using water displacement methods. Gas analysis was conducted every 6–12 h using Bluesens sensors for Hydrogen, Methane and Carbon Dioxide (Bluesens, Germany). Glucose analysis was carried out at 6 h intervals using a Glucose analyser (YSI 2700). The chemical oxygen demand (COD) of the substrate and effluent were carried out using COD cell test kits (Merck). pH of the suspended cell culture process was measured at 6 h intervals. Biofilm formed on the surface of the cartridges was observed under phase contrast microscopy (Zeiss). The molar hydrogen yield was obtained according to Equation (1).

$$HY = N_{H,F}/(N_{g,i} - N_{g,f}) \quad (1)$$

where HY is the molar hydrogen yield, $N_{H,F}$ is the cumulative hydrogen volume in moles, $N_{g,i}$ and $N_{g,f}$ represents the initial and final glucose concentration in each run respectively.

The novel reactor and control were comparatively assessed in terms of hydrogen fraction, duration of lag and exponential phases, total gas produced, chemical oxygen demand and yield of hydrogen. Changes between the novel reactor and the control with regards to the above parameters were expressed using Equation (2).

$$\text{Percentage change} = R_P - C_P/C_P \times 100 \quad (2)$$

where R_P is the reactor parameters and C_P is the control parameters of hydrogen fraction, fermentation time of lag and exponential phases, total gas produced, chemical oxygen demand and yield of hydrogen.

The cumulative volume of hydrogen produced was calculated according to Equation (3):

$$V_{H,i} = V_{H,i-1} + (V_{G,i} - V_{G,i-1}) \quad (3)$$

$V_{H,i}$ 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.

Microscopic analysis of sludge-alginate beads

The beads from fermentation batches were examined under scanning electron microscopy. The beads were placed in 3% buffered Gluteraldehyde for 2 h and washed in 0.05 M Sodium Cocodylate buffer twice for 5 min each. Sequential dehydration was performed with 10, 30, 50, 70, 90 and 100% ethanol for 10 min each. The beads were then dried in a Quorum K850 critical point dryer, mounted on carbon tape, attached to copper stubs and coated with gold in an Eiko IB-3 ion coater and viewed in a Zeiss EVO LS15 scanning electron microscope.

Results and discussion

Bioreactor design and implementation

In this study a novel supporting cartridge for cell immobilization has been designed and 3-D printed using Polylactic acid (PLA) filament. By designing and 3D-printing this component, there is a possibility to assess the efficiency of various configurations at low cost on process output in subsequent studies. The cartridge design and 3D-printing files have been made available as supplementary files. The tiny holes on the surface of these cartridge ensured that the alginate beads containing the microbial cells remained within the cartridges whilst having maximum exposure to the substrate. The cartridges are radially laid out on the vessel surface and also served as baffles thus promoting homogeneity during the wave mixing motion of the reactor. Four additional ports were included to accommodate additional sensors, making the system modular and expandable. The implemented reactor was low cost with readily available components on the market.

Heat and mass transfer efficiency

The reactor achieved a raise in temperature for 1 °C in 12 min. This time could be shortened by simply substituting a higher power heating blanket. A 58 s time period was recorded to re-stabilize pH during the mass transfer efficiency experiment. Both the heat and mass transfer capabilities are influenced by the mixing speed [17].

Fermentation

Assessment with immobilized cells

Assessment of the bioreactor was done using mixed cultures. The use of mixed cultures in dark fermentation has some advantages as they promote the formation of biomass granules, use up oxygen, or decompose complex organic matter thus aiding the formation of hydrogen [18]. In addition to these beneficial organisms found in a mixed culture there are also methanogens, homoacetogenes and lactic acid bacteria that compete for the glucose carbon source, their presence makes it necessary to pre-treat the anaerobic sludge to eliminate these microorganisms [19]. The novel bioreactor was designed primarily for fermentation processes conducted with immobilized cells as they show a greater efficiency in production of hydrogen and have the advantage of not being washed out of the bioreactor during continuous processes with short hydraulic retention times [20]. Fermentation process with immobilized cells gave cumulative volumes of 341 ml and 204 ml hydrogen and carbon dioxide respectively for the implemented reactor which was higher than the control with cumulative volumes of 196 ml and 36 ml hydrogen and carbon dioxide respectively under similar experimental conditions. The hydrogen yield was 0.280 mol H_2. mol^{-1} glucose and 0.213 mol H_2 mol^{-1} glucose for the implemented reactor and control respectively. This corresponds to a 31% increase in the hydrogen yield of the implemented reactor. The lag and exponential phases in the implemented reactor were significantly shorter, 16.5 h was required compared to the control where 25 h was observed (Fig. 3A). A peak hydrogen fraction of 37.82% and 28.03% was achieved in the implemented reactor and control respectively (Fig. 3A).

The glucose concentration reduced from 10 g/l to 0 and 2.40 g/l in the implemented and control reactors respectively after 12 h of fermentation (Fig. 3B). It was further observed that after 12 h of fermentation, immobilized beads in flask reactor began to float to the broth surface due to gas accumulation within the matrix and a poor agitation characteristic of flask cultures. These phenomena might have contributed to the incomplete metabolism of glucose with the consequential lower hydrogen production in flask reactor. The presence of biofilm formation on the cartridge wall of the implemented reactor was observed (Fig. 3C and D). Microscopic examination revealed the presence of rod shaped organisms. The chemical oxygen of the substrate was reduced from 12.7 g/l to 6.8 g/l and 7.6 g/l for the implemented bioreactor and the flask configuration respectively (Table 1). This indicated that the implemented reactor was 15.7% more effective than the flask reactor.

Comparative fermentation processes with the immobilized cells between the implemented reactor and the control flask done in replicate revealed three key findings. Firstly the lag phase for hydrogen production within the implemented bioreactor was shortened by 58% compared to the control. Secondly the maximum fraction of biohydrogen from the implemented reactor was 35% higher than that achieved in the control. Thirdly the volume of biohydrogen generated using the implemented bioreactor was 74% higher in the implemented bioreactor. The presence of biofilm on the surface of the cartridges might have been a crucial causative

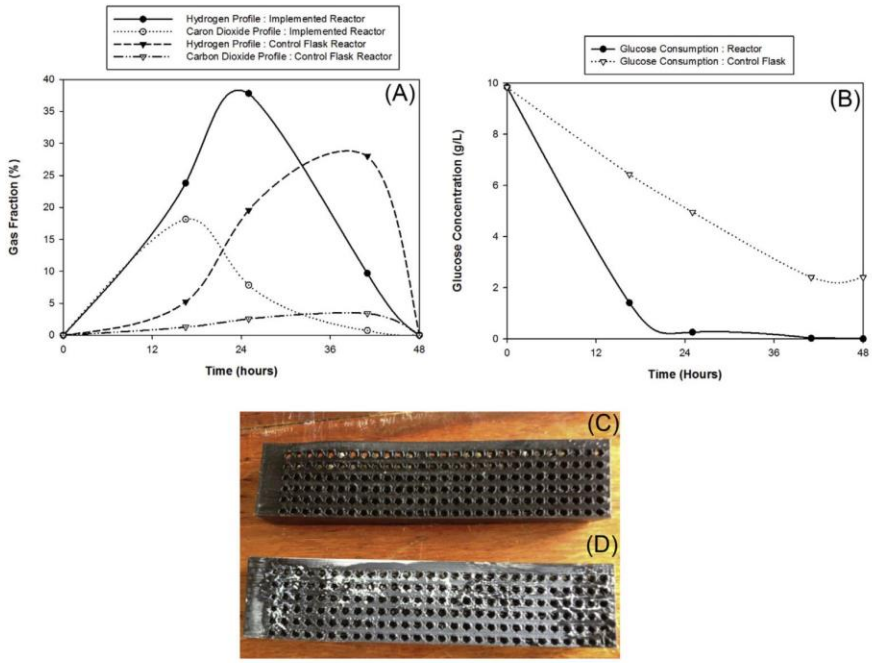

Fig. 3 – Comparative assessment of implemented bioreactor and control flask using immobilized microbial cells: (A) hydrogen and carbon dioxide profile for a 48 h fermentation period; (B) Glucose consumption profile; (C) cartridge before fermentation; (D) biofilm that formed on the surface of cartridge after fermentation.

Table 1 – COD for substrate and effluent from implemented reactor and control flask.

Sample (substrate)	COD (g/L)
Substrate	12.7
Effluent: implemented bioreactor	6.8
Effluent: control flask	7.6

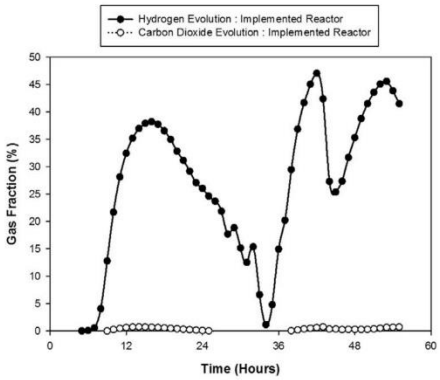

Fig. 4 – Hydrogen and Carbon Dioxide evolution from implemented reactor in real-time using immobilized cells.

influence to the enhanced hydrogen production efficiency within the implemented bioreactor. The relative superiority of the implemented bioreactor over the traditional culture system may be linked to the efficient mixing procedure with the immobilized cells support cartridge. The residual glucose in the flask reactor was 24%, thus an incomplete process.

Real-time monitoring of the implemented bioreactor using immobilized culture

Hydrogen production showed 3 main peaks, the first at 16 h (38% hydrogen), the second at 43 h (48% hydrogen) and the third at 53 h (46% hydrogen) (Fig. 4). The oscillations of hydrogen production observed in hydrogen profile may be linked to a series of factors amongst others such as the pH and hydrogen partial pressure and the inoculum source [2,21]. The pH of the reactor dropped during the fermentation from 6.8 to 4.41. A study carried out by Zhu and Yang [22] indicated that the enzymatic production was affected by the pH drift which in turn changes the metabolic shift of hydrogen production. Wang and Wan [23] suggested that the hydrogenase activity and the metabolic pathway are also affected by changes in pH when pH is not controlled. Hallenbeck and Ghosh [24] showed that the pathway leading to the production of lactate also becomes activated at an acidic pH to relieve acid stress thus reducing the hydrogen production. Hydrogen partial pressure shifts metabolisms to producing more intermediates, thus decreasing the overall hydrogen produced when its concentration in the reactor is excessively high [2]. The decline in hydrogen production from 15 to 34 h and 42 to 45 h could be a result of the hydrogen concentration in the reactor being markedly high causing metabolic shift. Mizuno et al. [25], showed that continuously sparging the bioreactor with nitrogen gas reduced the hydrogen partial pressure. Bastidas-Oyanedel et al. [26] explained that this in turn affected the functionality of lactate hydrogenase, NADH hydrogenase and homoacetogenesis reactions which contributed to the increase in hydrogen yield.

Assessment with suspended cells

The fermentation batch with suspended cells indicated that the rate of biohydrogen evolution was faster within the implemented bioreactor than that of the Erlenmeyer flask control (Fig. 5A). A peak hydrogen fraction of 54% and a residual glucose concentration of 0.7 g/l was obtained in the implemented reactor compared to a peak hydrogen fraction of 48% and a residual glucose concentration of 0.14 g/l in the flask reactor (Fig. 5A and B). The implemented reactor produced hydrogen maximally within the first 16 h compared to the flask reactor in which hydrogen production started after a 16 h lag period (Fig. 5A). The flask reactor showed a peak hydrogen fraction after 36 h (Fig. 5A). These data suggested that the novel bioreactor improved hydrogen production time by 60%. A high correlation was observed between glucose concentration and pH trends within the two batch processes (Fig. 5B). The completed degradation of glucose with suspended culture processes could be attributed to the greater availability of the substrate to the cells than in the

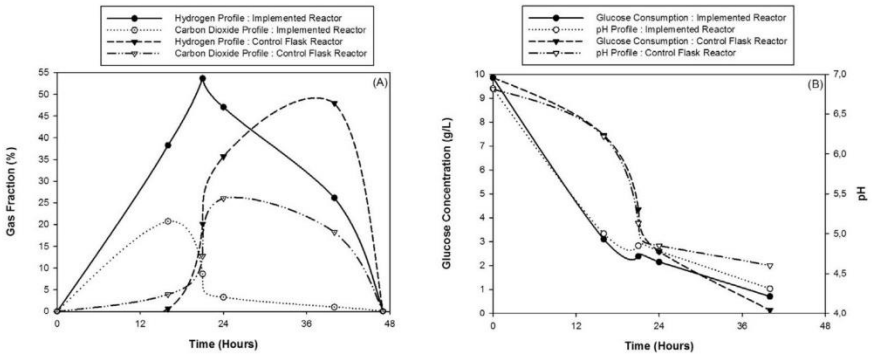

Fig. 5 – Comparative assessment of implemented bioreactor and control flask using suspended cells: (A) hydrogen and carbon dioxide profile for a 48 h fermentation period; (B) glucose consumption profile.

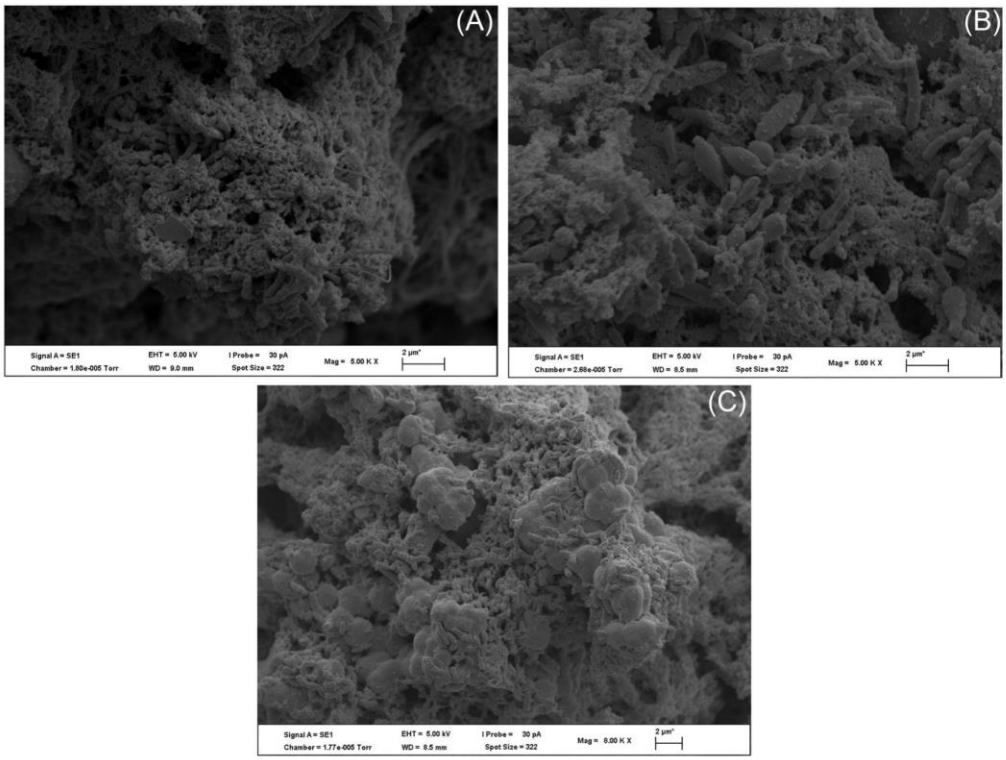

Fig. 6 – Scanning electron micrograph of a cross section from a sludge alginate beads (A) before fermentation at 5000x, (B) after fermentation from the shake flask control at 5000x, and (C) after fermentation from the implemented bioreactor cartridge at 8000x.

immobilized cells in the control flask. The pH decreased from 6.8 to 4.61 and 4.3 in the control flask and implemented bioreactor respectively within a 48 h (Fig. 5B). The pH decrease and glucose consumption showed a good correlation with hydrogen production profile within the implemented bioreactor and flask reactor respectively (Fig. 5A and B).

Microscopic analysis of sludge-alginate beads

Microscopic analysis of the alginate beads prior to fermentation showed the presence of rod shaped cells within the cross-links of the alginate matrix as indicated in Fig. 6A. The beads from the Erlenmeyer control (Fig. 6B) showed a non-uniform microbial growth compared to the beads from the implemented reactor which showed interactive growth between rod shaped cells proliferating on the surface of cocci shaped microorganisms (Fig. 6C).

Conclusion

The implemented bioreactor had an economical, robust and simple mechanical design, an easy operation, and a good mass transfer efficiency. The immobilized beads retained their integrity thus sustaining the microbial community structure under stringent agitation. The ability of this configuration to maintain microbial biomass makes it possible to facilitate short hydraulic times, which promote hydrogen production. The implemented reactor gave more insights on the process dynamics, this information is vital for an effective process scale up. The implemented reactor configuration and operation present great potentials to enhance the industrial production of biohydrogen with immobilized microbial communities.

Acknowledgements

The financial assistance of the National Research Foundation (NRF) (CPRR13091742710 and RSE14080184761) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at are those of the author and are not necessarily to be attributed to the NRF.

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Chapter 4

Adaptation of cell immobilization cartridge unit to continuous stirred tank reactor improves dark fermentative hydrogen production

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This chapter has been submitted to Renewable Energy with the title: Adaptation of cell Immobilization Cartridge unit to Continuous Stirred Tank Reactor Improves dark fermentative hydrogen production.

The manuscript is presented in the following pages:

Adaptation of cell Immobilization Cartridge unit to Continuous Stirred Tank Reactor Improves dark fermentative hydrogen production

M.B. Hassan Khan, E.B. Gueguim Kana*[1]

Discipline of Microbiology, School of Life Sciences, University of KwaZulu-Natal, Private Bag XO1, Scottsville, 3209 Pietermaritzburg, South Africa

Abstract

Challenges associated with biohydrogen production in a continuous stirred tank reactor (CSTR) using immobilized cells include bead floatation due to gas accumulation, excessive sheer stress and biomass washout in continuous processes. Thus, the need for a novel reactor configuration. In this study, a three-part cartridge structure was incorporated into a standard CSTR. The cartridge (46x40x300 mm) was made up of perforated hollow rectangular tubes aligned at 120° along the agitating shaft. The designed structure houses the immobilized inoculum and also served as impellers. This structure was fabricated using laboratory grade stainless steel. A comparative assessment of the cartridge and standard CSTR with suspended and immobilized mixed microbial culture was carried out. Fermentation process using the Immobilized Cells in Cartridge (ICC) showed an improved hydrogen yield of 3.9 fold and 2.8 fold compared to the Suspended Cells in Reactor (SCR) and Immobilized Cells in Reactor (ICR) processes. ICC decreased fermentation time by 52 and 65% compared to SCR and ICR respectively. Gompertz model coefficients showed a 98 and 37% improvement in the maximum hydrogen production rate (R_m) using the ICC compared to the SCR and ICR fermentations. Additionally, the ICC showed improved pH buffering capacity and complete glucose degradation. These findings demonstrate that the developed ICC concept holds innovative proficiencies to improve fermentative biohydrogen productivity, yield and process economics.

Keywords: Hybrid biogas bioreactor, Dark fermentation, Biohydrogen, CSTR, cell immobilization

[1] Corresponding author. Tel.: +27 332605527.
E-mail address: kanag@ukzn.ac.za (E.B. Gueguim Kana).

Highlights:

- The use of immobilized cells with the cartridge unit improved hydrogen yield
- The developed system prevented damage or destruction of the immobilized matrix
- Challenge of bead floatation associated with gas production was eliminated.
- Improved pH buffering capacity and complete glucose degradation was achieved.

1. Introduction

The environmental impact of fossil fuel usage ensues in increased greenhouse gas emissions and depletion of the ozone layer. Mitigation of these impacts can be achieved by harnessing an inexhaustible environmentally friendly low-cost energy source such as biohydrogen. Hydrogen is also the only known fuel that is free from carbon, and will therefore not contribute to acid rain, the greenhouse effect and ozone depletion upon combustion [1]. Combustion of hydrogen results in water vapour and energy making it the ideal innocuous fuel [1]. Hydrogen has a chemical energy of 142 MJ kg^{-1} per mass, this is a minimum of three times the equivalent of other chemical fuels [2]. The three core methods of biological hydrogen production include biophotolysis of water, photodecomposition of organic composites and fermentation of organic wastes [3]. Fermentation of organic wastes transpires at a higher rate and has extensive applicability and integration prospects [4, 5]. The dark fermentative hydrogen production bioprocess is sensitive to various physicochemical process parameters. These include inoculum type, preparation and state, temperature, hydraulic retention time (HRT), hydrogen partial pressure, pH, substrate type and bioreactor configuration and operation amongst other physicochemical influences [6]. Biomass retention, heat and mass transfer have significant impact of biohydrogen yield [3, 7, 8], both of which depend predominantly on bioreactor design [9]. The primary objective of a bioreactor is to provide a controlled microclimate optimal to cellular growth and/or product formation [10]. A suitable bioreactor for dark fermentation should maintain an adequate biomass concentration, efficient mixing with reduced shear stress, facilitate homogenous nutrient supply and be proficient at product formation and extraction, amongst other standard functions [10]. Biohydrogen production using a continuous stirred tank reactor (CSTR) was previously reported [11, 12, 13]. This configuration possesses an outstanding mixing ability, which plays a

significant role in maintaining homeostasis in the physicochemical parameters of a fermentation process. The mixing mechanism of the CSTR accommodates a conglomeration of substrates, particularly waste materials, ranging from highly viscous mixtures to heterogeneous composites with insoluble factions. The hydrodynamics and superior mass transfer of the CSTR are exceedingly commendable attributes of this configuration [11]. The intrinsic design of the CSTR is not compatible with protecting biomass washout during short HRT, which is essential for optimum hydrogen production, due to excessive sheer stress and biomass washout [3, 11, 14]. Less turbulent mixing systems with a greater potential to maintain immobilized biomass, viz. fixed bed reactors, up flow reactors, anaerobic granular sludge bed bioreactors and membrane reactors are alternative configurations commonly used in dark fermentative biohydrogen production [11]. However, impaired mass transfer, reduced substrate conversion rates and decreased hydrogen production have been identified as major limitations in these immobilized reactors [15], thus accentuating the need for a novel bioreactor configuration for biohydrogen production [16].

Cell immobilization is the physical restriction or isolation of whole cells to a distinct area to carry out a chosen purpose [17]. Enhanced hydrogen production was reported using immobilized cell systems [18]. Immobilization influences the microbial inoculum's tolerance to metabolic strain in terms of temperature, pH, organic loading rate and hydraulic retention time [3, 18]. The major methods for microbial cells immobilization include encapsulation, adsorption and polymer-based entrapment [18]. Adsorption involves the physical attachment of bacterial cells to a support matrix via bonding of positive and negative charges [18], an example is the use of coir in a packed bed reactor [19]. The ionic charge of the reaction mixture impacts the integrity of the attachment, therefore a change in the pH may affect the immobilization process. Encapsulation is a process wherein bacterial cells are enclosed within a semi-permeable membrane [18], e.g. the use of polyvinylidene fluoride (PVDF) in a serum glass bottle [20]. This technique minimises the escape of bacterial cells into the reaction mixture, reducing downstream processing of effluent [18]. The entrapment of bacterial cells, the most common and preferred technique, entails the incorporation of bacterial cells into a porous matrix [18]. The matrix may be biodegradable, e.g. alginate or agar, or non-biodegradable, e.g. acrylic, latex or silicone [18]. The most suitable choice depends on various factors, namely, the nature of the fermentation,

the physicochemical characteristics of the substrate, metabolism of inoculum and the bioreactor chosen to orchestrate the fermentation process. Zhao *et al.* [21] reported a 41% increase in the hydrogen production rate compared to the control using mycelium pellets to immobilize *Clostridium* species in a CSTR. Adsorption and entrapment on substances such as polyurethane and silicone are commonly used in fluidized bed reactors [22, 23]. Granular activated carbon may be used independently or as an auxiliary ingredient in immobilization matrices [24, 25]. Immobilized cell technology was reported in dark fermentative hydrogen generation as a means of increasing productivity via cell retention. For example, an improvement of 71% on the rate of hydrogen production was reported using immobilized pure cultures [26]. Similarly, immobilized mixed microbial cultures have shown an improvement in the rate of hydrogen production within the range of 52 - 213% [13, 27]. Enhanced substrate conversion and pH buffering are benefits of immobilization that aid in hydrogen productivity [26, 28]. The mechanical strength of alginate matrices is susceptible to collapse, and gas producing immobilized beads are prone to float as gas production progresses [27], which ultimately impedes mass transfer. Salem *et al.* [13] suggested that the method of immobilization must harmonise with the bioreactor design to improve fermentation dynamics. Inappropriate matrices could impede mass transfer and have an adverse effect on product formation. Moreover the collusion of the carrier against the walls or the reactor may cause the microorganisms to become dislodged and lost into the fermentation broth [13]. These observations suggest the need to investigate for more appropriate bioreactor configuration for biohydrogen production using immobilized cells.

In this study a novel cartridge structure for cell immobilization is designed and adapted to the CSTR, **I**mmobilized **C**ells in **C**artridge (**ICC**). The developed reactor is comparatively assessed against the standard CSTR on dark fermentations using **S**uspended **C**ells in **R**eactor (**SCR**) and **I**mmobilized **C**ells in **R**eactor (**ICR**).

2. Materials and methods

2.1. Bioreactor modification and design of the cartridge unit

The standard CSTR reactor used in this study was a 13 L INFORS HT Labfors 3 (Infors AG, Switzerland). The CSTR was modified by the addition of a cell immobilization cartridge (Figure 1). The cartridge was designed using the CAD modelling software OpenSCAD version 2015.03 (http://www.openscad.org). It constituted of three rectangular perforated ducts of 46 mm x 40 mm x 300 mm size (Figure 1A). The pore size was 3 mm in diameter (Figure 1A). The total volume of the cartridge was 1656 ml. The three ducts were aligned at 120° around a central pipe with an external diameter of 15 mm, followed by a shift of +7,5 mm on the x-plane and -15 mm on the y-plane (Figure 1B). This was necessary in order to minimise the outer rotation diameter of the cartridge, whilst ensuring the placement of the reactor shaft (Figure 1B). The top and bottom cover plates enclosed the structure at both ends (Figure 1C). These cover plates have a central opening of 15.5 mm to enable the passage of an agitation shaft. Grub screws held the complex together and attached it to the agitating shaft simultaneously (Figure 2B). Dynamic stability was achieved by ensuring that the cartridge fitted precisely around the reactor shaft. Other designed considerations were: maximising the cartridge size to allow a variety of inoculum concentrations, promoting enhanced exposure between inoculum and substrate and ensuring that the cartridge was able to integrate within the standard reactor unobtrusively during agitation. The cartridge structure was removable and could be interchanged with other standard impellers. The material used to fabricate the cartridge unit was a 1.2 mm thick stainless steel sheet (316-2B) (Figure 1C).

The developed reactor was comparatively assessed against the standard CSTR on dark fermentations using suspended and immobilized mixed microbial cells.

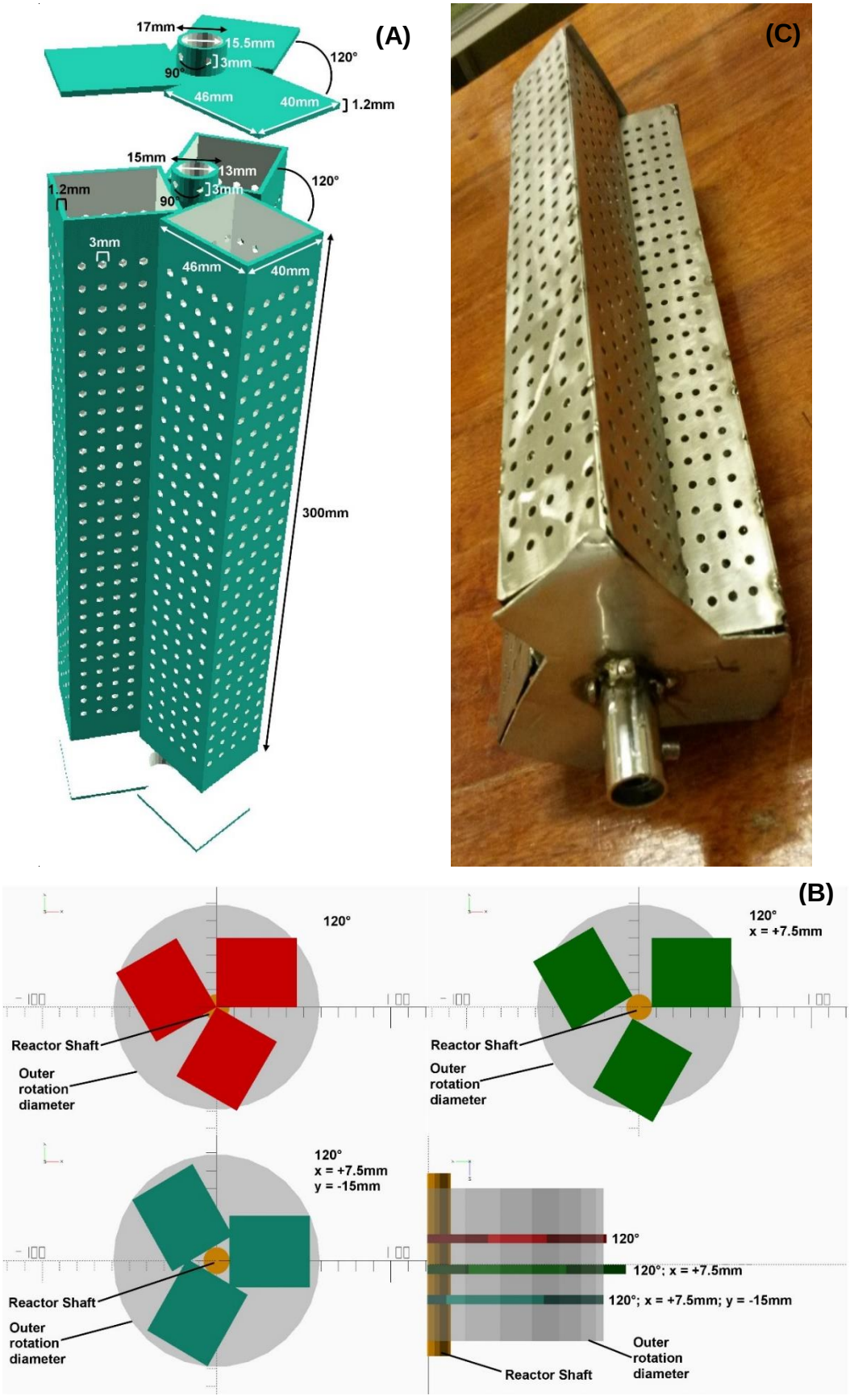

Figure 1: Cartridge turbine for housing of immobilized microorganisms: (A) design and dimensions; (B) Placement of three ducts that from the cartridge; (C) Fabricated cartridge using stainless steel.

2.2. Assessment of the developed bioreactors for fermentative biohydrogen production

2.2.1. Inoculum and culture medium preparation

The inoculum consisted of anaerobic sludge acquired from Darville wastewater treatment facility in Pietermaritzburg, South Africa. A heat pre-treatment was carried out at 121°C for 10 min following a 24 hour incubation at pH 8 at 25°C [29] to deactivate hydrogen consumers. Sodium alginate (Sigma) was mixed with the pre-treated anaerobic sludge to make a final 3% alginate solution, containing a 20:80 alginate to sludge ratio. 0.2% granular activated carbon was added to increase bead porosity [25]. The alginate sludge mixture was then trickled through a peristaltic pump (Watson-marlow 503U) into a 0.2 M $CaCl_2$•2H_2O solution which enabled the alginate to cross-link into small beads 3 mm in diameter. The fermentation medium consisted of 1.5 g L^{-1} KH_2PO_4, 2 g L^{-1} $(NH_4)_2SO_4$, 0.157 g L^{-1} $FeCl_2$•4H_2O, 0.1 g L^{-1} $CaCl_2$•2H_2O, 0.085 g L^{-1} $MnCl_2$•4H_2O, 0.178 g L^{-1} $ZnSO_4$•7H_2O, 0.072 g L^{-1} Na_2MoO_4•2H_2O and 10 g L^{-1} $C_6H_{12}O_6$ [29].

2.2.2. Bioreactor operation

Hydrogen was produced with an 8 litre working volume utilising a 10% inoculum size (800 ml). Operational conditions of 37°C and 180 rpm were used for temperature and agitation respectively with an initial pH of 6.8.

Three fermentation setups were carried out in batch mode. These encompassed, (1) Suspended Cells in Reactor (SCR), wherein suspended cells was placed directly into the substrate. (2) Immobilized Cells in Reactor (ICR), wherein immobilized cells were placed directly into the substrate. (3) Immobilized Cells in Cartridge (ICC), wherein immobilized cells was placed equally into the three rectangular perforated ducts of the cartridge structure. The setup 1 and 2 were carried out in 13 L Infors bioreactor equipped with two Rushton turbine impellers (Figure 2A). The setup 3 used a similar bioreactor vessel, but without impellers as the cartridge system ensured the mixing process (Figure 2B).

The reactor was sparged with nitrogen gas for 6 min directly prior to each start-up. Fermentations were run for the duration of hydrogen production.

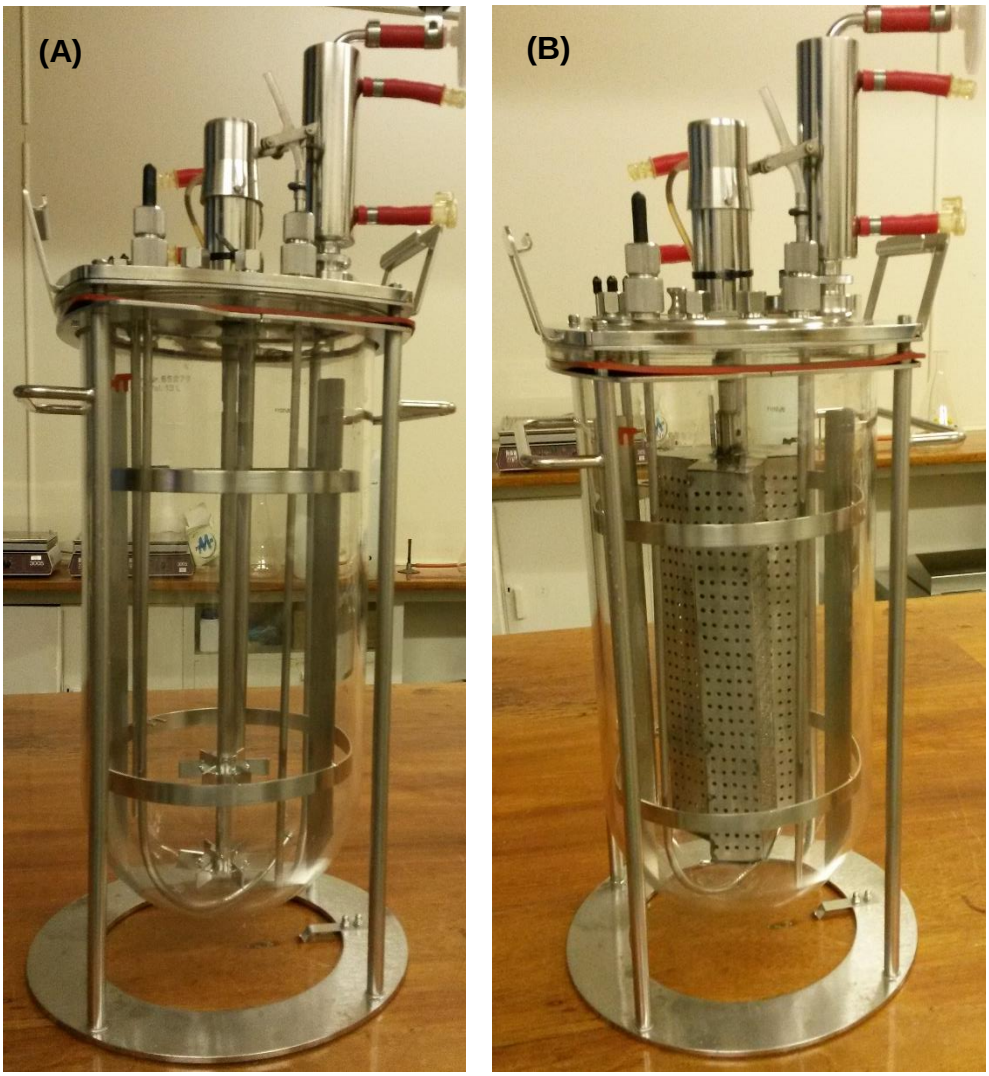

Figure 2: Continuous stirred tank reactor: (A) before modification, Rushton turbine impellers present; (B) after modification with removable cartridge turbine

2.3. Analytical methods

2.3.1. Gas and liquid phase

The total biogas produced was monitored in real time using f-lab software [30] via Bluesens sensors for Hydrogen, Methane and Carbon Dioxide (Bluesens, Germany) and a MILLIGASCOUNTER® (Ritter, Germany) under standard temperature and pressure. The molar hydrogen yield was calculated according to equation (1).

$$HY = N_{H,F} / (N_{g,i} - N_{g,f}) \quad (1)$$

Where HY is the molar hydrogen yield, $N_{H,F}$ is the cumulative hydrogen volume in moles, $N_{g,i}$ and $N_{g,f}$ represents the initial and final glucose concentration in moles, for each run respectively.

The cumulative volume of hydrogen produced was calculated according to the equation (2)

$V_{H,i} = V_{H,i\,-1} + C_{H,i}\,(V_{G,i} - V_{G,i\,-1}) + V_H\,(C_{H,i} - C_{H,i\,-1})$ (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 $C_{H,i\,-1}$ are the fraction of hydrogen gas in the headspace of the bioreactor in the current and previous intervals, and V_H the total volume of the headspace in the bioreactor.

The modified Gompertz model was used to assess the kinetics of biohydrogen production for SCR, ICR and ICC batch fermentations using equation (3).

$$H(t) = P \cdot exp\left\{-exp\left[\frac{R_m \cdot e}{P}(\lambda - \mathrm{t}) + 1\right]\right\} \quad (3)$$

Where *H(t)* represented the cumulative volume of hydrogen production (ml), *P* is the hydrogen production potential (ml), R_m is the maximum hydrogen production rate (ml h^{-1}), λ is the lag-phase time (h), *t* is incubation time (h), and *e* = 2.718.

The parameters P, Rm and λ were derived using the least squares method using CurveExpert V1.5.5 software.

Glucose and pH were monitored at 6 hour intervals and analysed using a Glucose analyser (YSI 2700) and a pH meter (Crison) respectively. The chemical oxygen demand (COD) of the substrate and effluent was assessed using COD cell test kits (Merck).

The SCR, ICR and ICC batch fermentations were comparatively assessed in terms of total gas produced, hydrogen fraction, cumulative hydrogen volume, total fermentation time, hydrogen yield, glucose consumption, pH change and COD reduction. The percentage differences between some of these process modes was expressed using equation (4).

Percentage change = $(C_p\text{-}R_p)/R_p$ x 100 (4)

Where **C**p is the IC**C** parameters and **R**p is the SC**R** or IC**R** parameters. Parameters include hydrogen fraction, total fermentation time, maximum hydrogen production rate, exponential hydrogen production phase, glucose consumption, pH change and COD reduction.

2.3.2. Microscopic inoculum analysis

The pre-treated inoculum anaerobic sludge, sludge-beads and the effluent beads from the SCR and ICC fermentations were observed under scanning electron microscopy. The pre-treated anaerobic sludge was homogenised and a single drop placed onto a filter and air dried within a fume hood (ESCO Frontier). The filter disk was then affixed to a copper stub via carbon tape and coated with gold in an Eiko IB-3 ion coater. The beads were placed in 3% buffered Gluteraldehyde for 2 hours and washed in 0.05 M Sodium Cocodylate buffer twice for 5 min each. Sequential dehydration was performed with 10, 30, 50, 70, 90 and 100% ethanol for 10 min each. The beads were then dried in a Quorum K850 critical point dryer, mounted on carbon tape, attached to copper stubs and coated with gold in an Eiko IB-3 ion coater and viewed under a Zeiss EVO LS15 scanning electron microscope [29].

3. Results and discussion

3.1. Bioreactor design and implementation

Stainless steel 316-2B, was used for cartridge fabrication. It has a high resistance to corrosion in chloride environments [31] and can withstand repeated cycles of heat sterilization at 121°C for 20 min, required for bioreactor components. The perforations and the orientation of the cartridges maximise the exposure of the cells to the liquid substrate. The structure and alignment of the cartridges, together with the baffles promoted turbulence and prevented a vortex formation. The cartridges were attached to each other at 120° to maximise the volume of the cartridges whilst ensuring stable agitation without damaging the various probes. The rotation movement of the cartridge enhanced mass and heat transfer with reduced shear damage on the immobilizing matrix. This improved the reusability of immobilized cells. This configuration could also be advantageous for processes with short hydraulic retention times, and would therefore benefit hydrogen fermentations [32]. Additionally, stabilizing the inoculum beads in the cartridge structure also prevented them from clogging the sensors or blocking the sampling ports in the bioreactor, thus enhancing process monitoring.

3.2. Comparative assessment of SCR, ICR and ICC for hydrogen production, glucose consumption and pH change

The SCR, ICR and ICC fermentation process modes were comparatively assessed. The total biogas volume from SCR, ICR and ICC were 1209, 1359 and 8210ml respectively with maximum biohydrogen fractions of 42.93, 46.92 and 30.30% (Figure 3A) corresponding to hydrogen volumes of 275.56, 383.16 and 1402.21ml (Figure 3B) respectively. The ICC system showed an improvement of 6.8 fold and 6 fold for the accumulated total biogas compared to SCR and ICR, indicating a greater degree of biological activity. Furthermore the ICC showed an increase in the cumulative biohydrogen volume of 5.1 fold compared to the SCR and 3.7 fold compared to the ICR. Despite the lower hydrogen fraction observed in ICC, the fermentation process in this system lasted for 25 hours against 52 hours in SCR and 71 hours in the ICR and resulted in an overall higher yield in ICC compared to both SCR and ICR processes (Figure 3A). The ICC reduced fermentation time by 52% and 65% compared to the SCR and ICR processes respectively (Table 1). Hydrogen yields were 0.288, 0.400 and 1.13 mol H_2 mol^{-1} glucose for the SCR, ICR and ICC processes respectively. This amounts to a 3.9 fold and 2.8 fold increase in the ICC compared to the SCR and ICR processes respectively.

A combination of a high hydrogen fraction with the low cumulative hydrogen volumes observed in the SCR and ICR processes could be indicative of hydrogen production inhibition by high hydrogen partial pressure [6, 15]. A high hydrogen concentration inhibits acetogenic bacteria that produce hydrogen [33]. The use of the cartridges created a greater degree of turbulence, thus preventing supersaturation of hydrogen gas in the liquid phase, therefore maintaining a low hydrogen partial pressure and preventing its negative effect on hydrogen production [6]. Although all processes were operated at the same mixing rate (180 rpm) the ICC process retained a greater mixing efficiency. Doran [34] states that replacing Rushton turbines with larger diameter turbines substantially reduces fluid compartmentalization and improves bulk mixing.

The modified Gompertz model kinetic coefficients are shown in Equations 5, 6 and 7 for SCR, ICR and ICC respectively

$$H(t) = 276 \cdot exp\left\{-exp\left[\frac{125 \cdot e}{276}(9{,}8 - \mathrm{t}) + 1\right]\right\} \qquad (5)$$

$$H(t) = 384 \cdot exp\left\{-exp\left[\frac{181 \cdot e}{384}(13{,}8 - \mathrm{t}) + 1\right]\right\} \qquad (6)$$

$$H(t) = 1532 \cdot exp\left\{-exp\left[\frac{248 \cdot e}{1532}(12{,}8 - \mathrm{t}) + 1\right]\right\} \qquad (7)$$

The regression curves gave coefficients of determination (r^2) values of 0.99, hence the models satisfactorily illustrated biohydrogen production for SCR, ICR and ICC fermentations. Table 1 shows the P, R_m and λ values for the SCR, ICR and ICC processes. The maximum hydrogen production rate (R_m) was 125, 181 and 248ml h^{-1} for the SCR, ICR and ICC processes respectively. This indicates that the ICC made a 98% improvement on the SCR process and a 37% improvement on the ICR process. The lag time (λ) of the SCR processes was the shortest (9.8 h), as was expected, due to the ease of availability of substrate to the cells. The ICC design was able to reduce the lag time (λ) by one hour compared to the ICR process (12.8h and 13.8h respectively), resulting from an improved mass transfer within the ICC. The hydrogen production potential (P) of the SCR, ICR and ICC processes were 276, 384 and 1532 ml respectively, thus 5.6 and 4 folds improvements by the ICC batch fermentation compared to SCR and ICR respectively. The lag time duration in dark fermentation under mesophilic conditions using anaerobic sludge are reported in the range of 4.29 h and 69.3 h [35, 36]. Lag times may fluctuate depending on pre-fermentation inoculum concentration, type and complexity of the substrate used. Short lag times do not necessarily result in a high Rm and P values. Kargi *et al.* [35] observed an R_m value of 1.53 ml h^{-1} and a P value of 98.1ml compared to Cakır *et al.* [36] with R_m and P values of 1.38ml h^{-1} and 125ml. Nath *et al.* [37] used a pure culture of *Enterobacter cloacae* DM11 and showed a P value of 927.64ml with a lag time of 2.05h and an Rm value of 17.65 ml h^{-1}. The maximum hydrogen production rate (R_m) and hydrogen production potential (P) are influenced by the substrate concentration, inoculum type and could vary depending on the favourability of the conditions within the reactor, i.e. reactor pH, VFA accumulation and hydrogen partial pressure. Agitation and mass transfer influence hydrogen production potential (P), maximum hydrogen production rate (R_m) and lag time (λ). The physicochemical conditions of fermentation like, the

inoculum/substrate type, methods of pre-treatment, concentration and physical state alongside constant pH and temperature conditions contribute to fluctuations in the P, R_m and λ.

The exponential time of hydrogen production lasted 6 hours for the SCR and ICR processes and 11 hours for the ICC process (Figure 3B). The rate of glucose degradation was proportional to hydrogen production in all three fermentation process. High glucose consumption rates were observed during the first 12 hours of fermentation in SCR and ICR, and 18 hours for ICC. This corresponded to high and stable production of hydrogen in these processes. Incomplete glucose degradation was observed in the SCR and ICR processes as the hydrogen production ceased after 52 and 71 hours respectively and these processes were terminated (Figure 3C). ICC process had a uniform rate of glucose consumption for the duration of fermentation, reducing the glucose concentration to a residual value of 0.049 g L^{-1}. A residual glucose concentration of 2.3g L^{-1} was obtained for SCR and ICR processes at the end of fermentation process unlike the ICC process mode. ICC process mode improved the glucose consumption by 29% compared to SCR and ICR processes. Fermentations were initiated at pH of 6.8, and a steeper decline of this value was observed in SCR and ICR compared to ICC over time. The final pH of the effluents were 4.35, 4.68 and 4.8 for the SCR, ICR and ICC processes respectively (Figure 3D). Thus, pH change was 19 and 10% lower in ICC compared to SCR and ICR respectively. This indicates that the ICC concept has buffering potential, which may have contributed to the enhanced hydrogen production. Hu *et al.* [38] indicated that the incomplete substrate degradation and consequent lower hydrogen yields may be attributed to a decrease in pH from acids production.

Table 1: Percentage change and fold improvements of the immobilized cells in cartridge (ICC) processes compared to the suspended cells in reactor (SCR) and immobilized cells in reactor (ICR) processes

Parameters	**Suspended cells in reactor (SCR)**	**Immobilized cells in reactor (ICR)**	**Immobilized cells in cartridge (ICC)**	**% Change of ICC to**		**Fold improvement of ICC to**	
				SCR	**ICR**	**SCR**	**ICR**
Total Gas volume (ml)	1209	1359	8209,5			6,8	6,0
Max. H_2 fraction (%)	42,93	46,92	30,30	-29	-35		
H_2 Volume (ml)	275,56	383,16	1402,21			5,1	3,7
Time (h)	52	71	25	-52	-65		
HY (mol H_2 mol^{-1} glucose)	0,288	0,4	1,13			3,9	2,8
Exponential H_2 production phase (h)	6	6	11	83	83		
Glucose degradation (g)	7,7	7,7	9,951	29	29		
pH change	2,46	2,21	1,997	-19	-10		
COD reduced (g L^{-1})	5,361	5,329	5,914	10	11		
P (ml) _ H_2 production potential	276	384	1532			5,6	4,0
Rm (ml h^{-1}) _ max. H_2 production rate	125	181	248	98	37		
λ (h) _ lag phase time	9,8	13,8	12,8	31	-7		

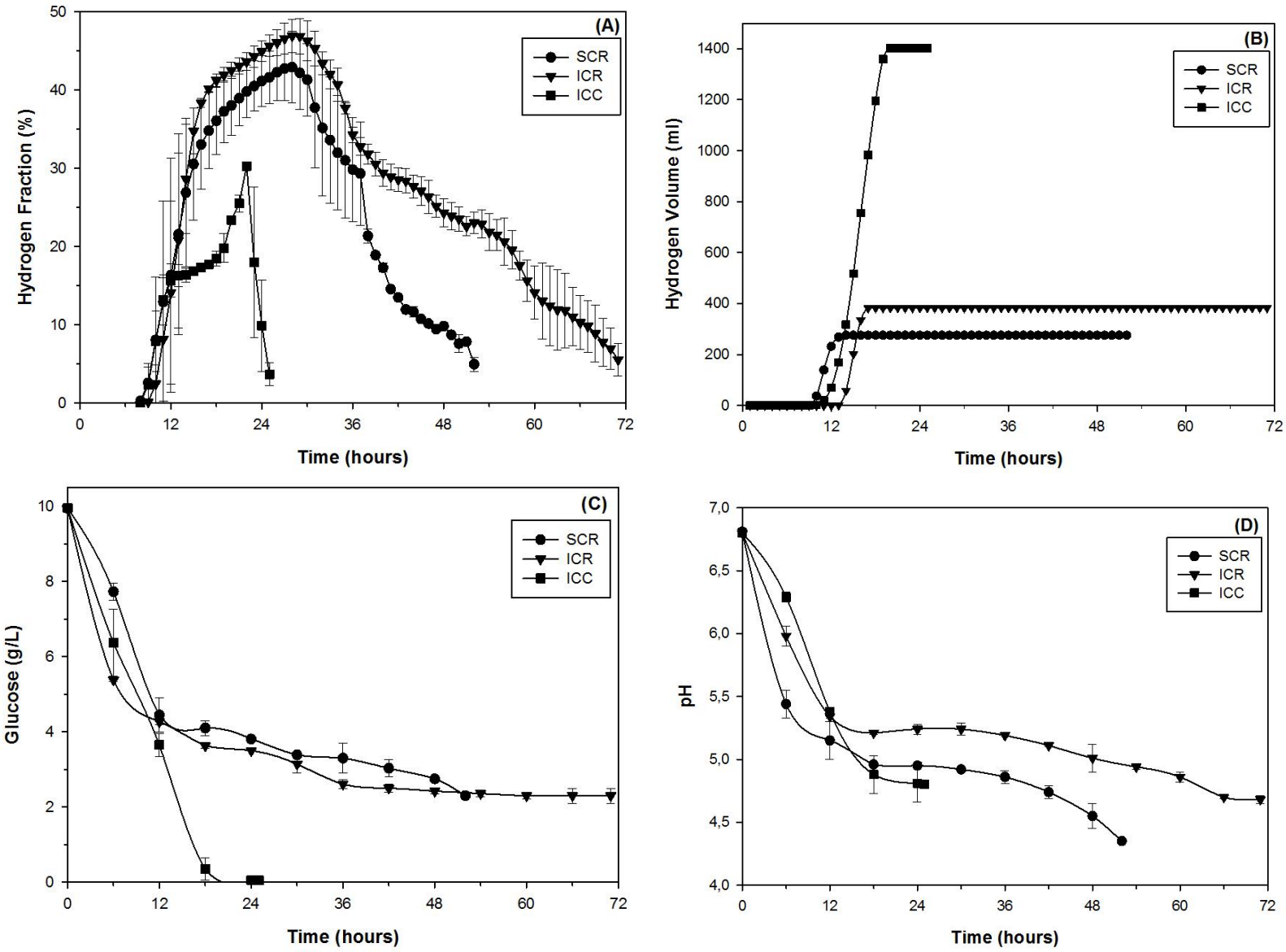

Figure 3: Comparative assessment of standard CSTR operated with SCR and ICR and the ICC design. Duration of fermentation varied. (A) Hydrogen fraction profiles; (B) Cumulative hydrogen volume profiles; (C) Glucose degradation profiles; (D) pH evolution profiles. (●: SCR; ▼: ICR; ■: ICC)

3.3. Chemical oxygen demand (COD)

The chemical oxygen demand of the substrate was reduced in all the three fermentations (Table 2). SCR, ICR and ICC fermentations reduced the COD of the substrate by 5,36; 5,33 and 5,91 g L^{-1} respectively. The ICC process had reduced the COD of the effluent by 10,3% and 11% more than the fermentation with SCR and ICR respectively. COD reduction and hydrogen production have a proportional relationship [38]. Bioreactor pH has an impact on COD reduction [38]. The wastewater and sludge had a COD of 12,73 and 15,3 g L^{-1} respectively (Table 2).

Table 2: Chemical oxygen demand of the substrate and effluent of the SCR, ICR and ICC processes, wastewater substrate and sludge and the COD reduction of the respective fermentation runs.

Sample	**Chemical oxygen demand (COD) (g L^{-1})**		
	Substrate	**Effluent**	**Reduction**
SCR	12,991	7,63	5,361
ICR	12,734	7,405	5,329
ICC	12,734	6,82	5,914
Wastewater substrate	12,734	12,734	-
Sludge	15,3	15,3	-

3.4. Electron microscopy (SEM) of sludge-alginate beads

The anaerobic sludge used in the fermentations showed the presence of various cocci and rod shaped microorganisms (Figure 4), as well as non-microbial components. Alginate beads from ICR and ICC process modes were observed. Beads from ICC mode retained better structural integrity compared to ICR beads, which were fractionated into various sizes. For the ICR processes, clusters of microbial cells were present in the ruptured beads (Figure 5), though majority of the surface area revealed the porous nature of the sodium alginate matrix. The beads from the ICC processes had fused via a cream coloured film, acquiring the shape of the cartridge. The cream coloured film showed a very dense population of rod shaped microorganisms approximately 2 µm in length (Figure 6A). The surface of the ICC beads had a uniform spread of varying microorganisms, ranging from small cocci, 1-2 µm in diameter, to rod microorganisms, 2-3 µm in diameter, vibrio microorganisms, 3 µm in length can

also be seen (Figure 6B). Micro cracks found sporadically on the surface of some ICC beads show the alginate cross links abundant in a rod shaped organisms, some of them appear to be joined and may represent *Diplobacillus* or *Streptobacillus* conformations (Figure 6C). A cross section of an ICC bead is shown in Figure 7D. In this micrograph some rod shaped microorganisms appear to be amidst various steps that occur in mitosis (Figure 6D). In light of this being an area that was completely enclosed during fermentation, it may indicate improved mass transfer in the ICC process to have cells appear to be in active stages of growth.

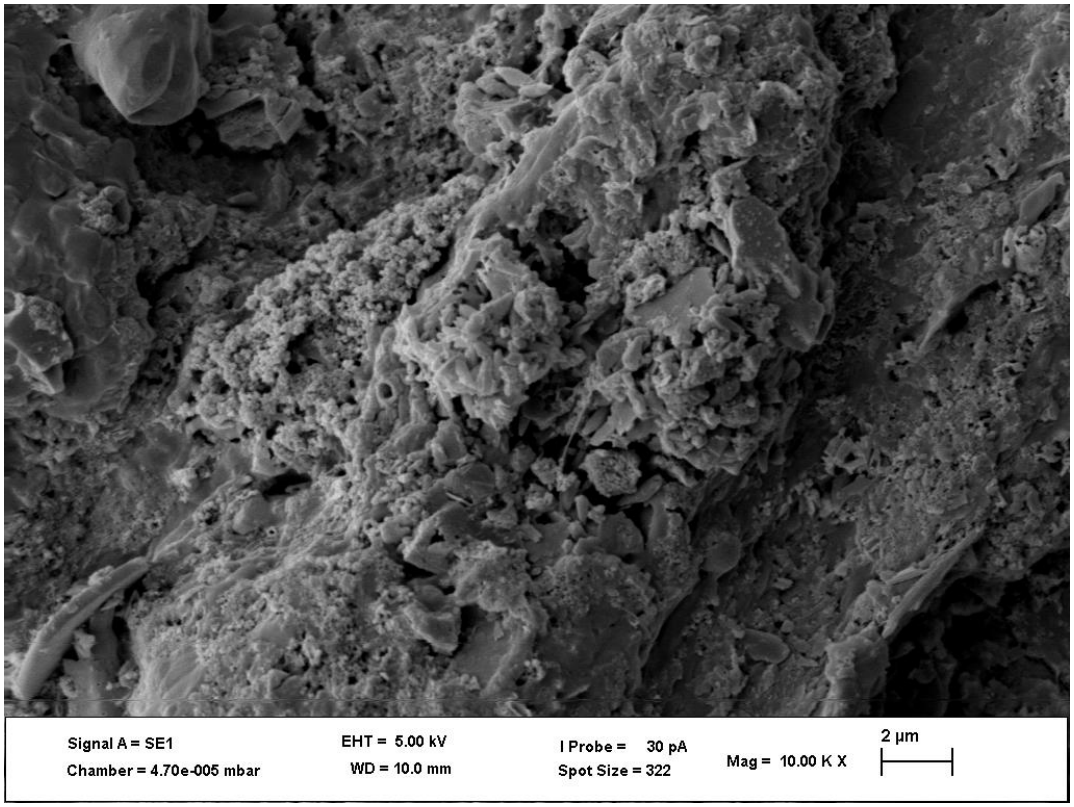

Figure 4: Anaerobic sludge prior to fermentation with rod shaped microorganisms present at 10 000x

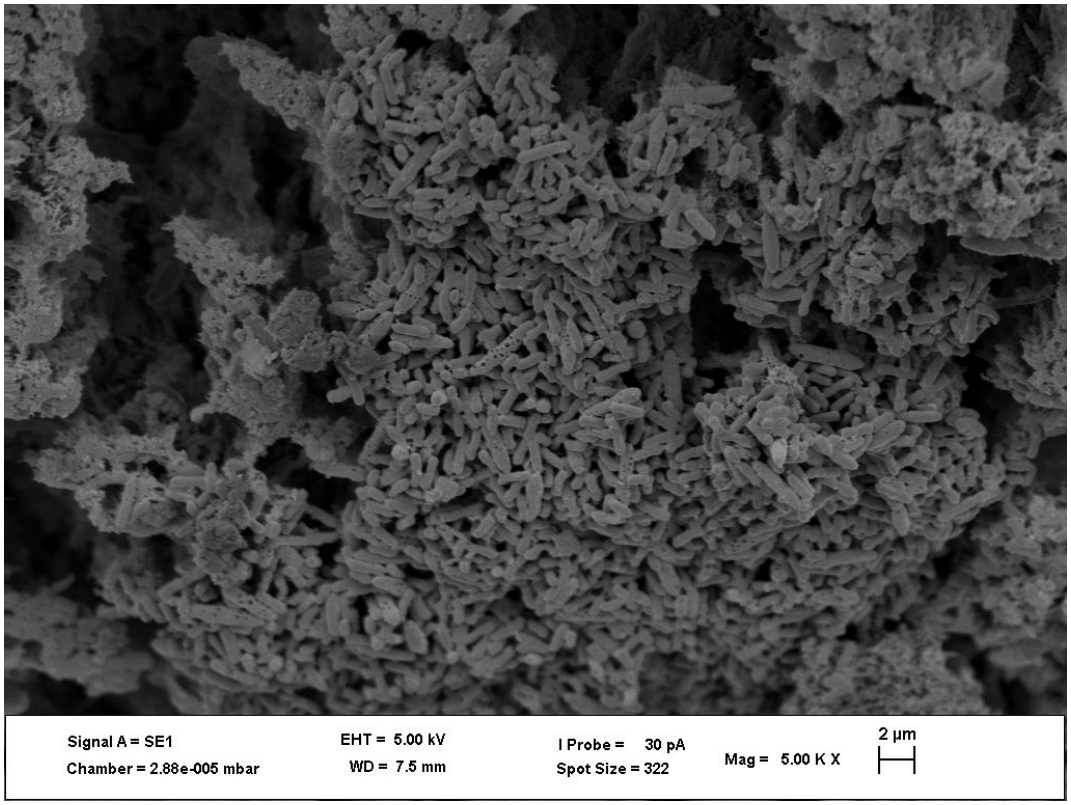

Figure 5: Sludge-alginate beads after ICR fermentation with microorganisms present in fragmented beads at 5 000x

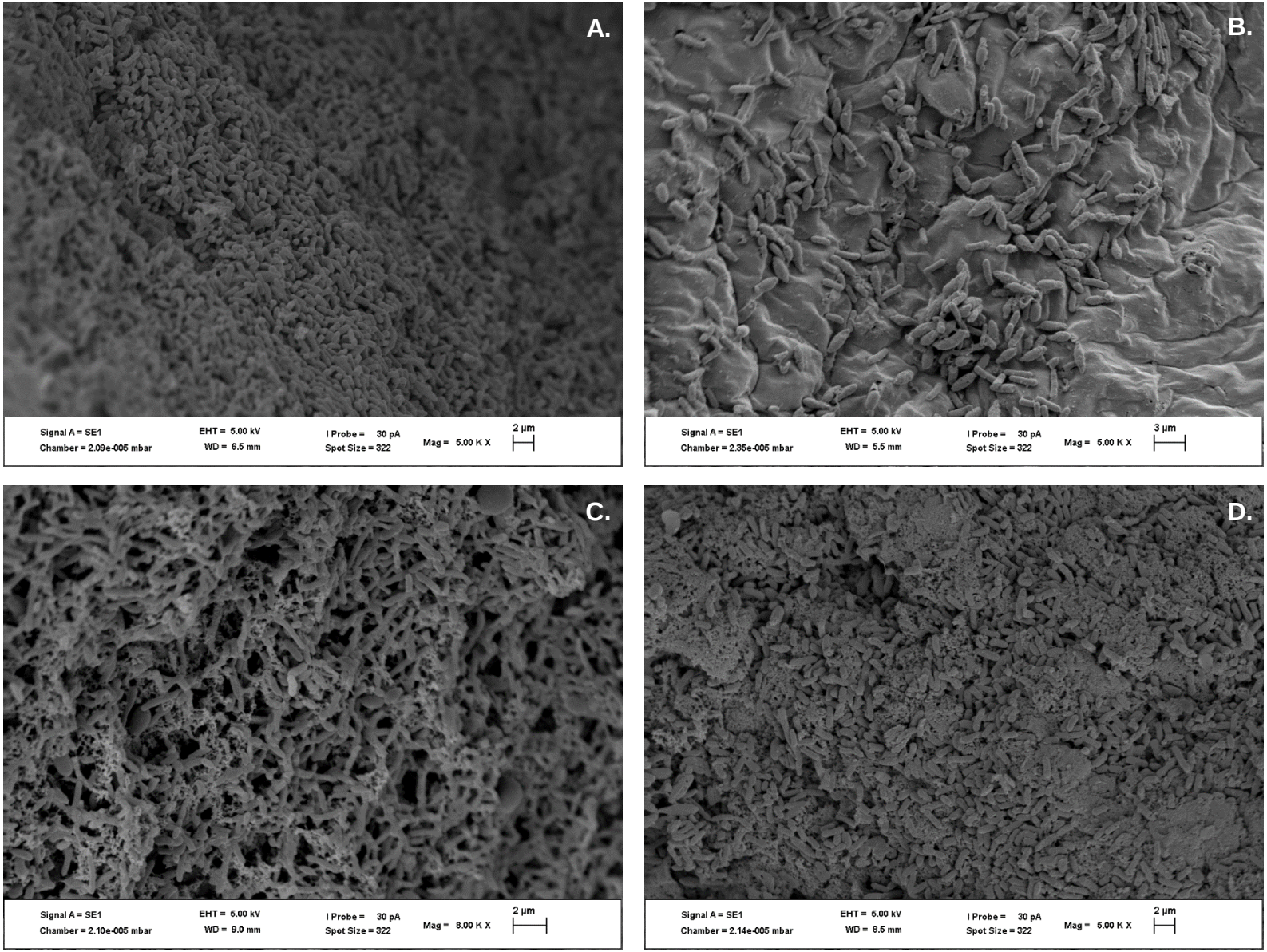

Figure 6: Sludge-alginate beads from within ICC fermentation: (A) film between fused beads at 5 000x; (B) exterior surface at 5 000x; (C) natural chasm on surface at 8 000x; (D) cross section of interior surface of beads fractured during SEM preparation at 5 000x

4. Conclusion

To address the challenges associated with the use of immobilized cells for biohydrogen production in a CSTR, a novel reactor configuration was developed using a cartridge system and comparatively assessed with the standard CSTR. The ICC process improved hydrogen yield by 3.9 and 2.8 folds and reduced fermentation time by 52 and 65% compared to the SCR and ICR process respectively. Kinetics studies using the modified Gompertz model revealed that the ICC process had a 4 fold increase in hydrogen production potential (P) compared to the ICR process. Complete glucose degradation was observed in the ICC process, which showed a 29% improvement compared to SRC and ICR processes. These findings indicate that the developed ICC concept holds innovative proficiencies to improve fermentative biohydrogen productivity, yield and process economics.

Acknowledgements

The financial assistance of the National Research Foundation (NRF) (CPRR13091742710 and RSE14080184761) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at are those of the authors and are not necessarily to be attributed to the NRF.

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Chapter 5

Conclusions and recommendations for further research

5.1. Conclusions and implications

This study designed, implemented a novel bioreactor configuration. This novel reactor was assessed on various fermentative biohydrogen production processes. Based on the findings, the following conclusions were drawn:

5.1.1. The novel 2L lab scale bioreactor was comparatively assessed with an equal volume shake flask reactor. Dark fermentation process with immobilized cells using the novel 2L lab scale bioreactor showed a reduced lag time of 58%, and an increased biohydrogen volume of 74% compared to the shake flask reactor. The novel reactor configuration showed a substantial improvement in hydrogen production.

5.1.2. The 13L novel bioreactor was compared with the standard CSTR under different fermentation modes. The standard CTSR was operated with suspended cells (SCR) and immobilized cells (ICR) and the novel reactor used immobilized cells (ICC). ICC showed a 5,1 fold improvement in the hydrogen volume, a 52% reduction in the total process time and a 3,9 fold increase in the hydrogen yield compared to SCR. In comparison with ICR, ICC showed a 3,7 fold increase in the hydrogen volume, a 65% reduction in the total process time and a 2.8 fold increase in the hydrogen yield.

5.1.3. ICC showed marked improvements in biohydrogen production compared to SCR and ICR. The study demonstrated that the cartridge concept was capable of improving hydrogen production with immobilized cells.

5.2. Recommendations for future studies

5.2.1. Further comparisons with the newly designed reactor and other configurations will provide insights into optimal bioreactor configuration to maximize hydrogen production.

5.2.2. Operating the novel reactor configuration using fed-batch or continuous modes would provide insights about the effect of hydraulic retention time on biomass retention and hydrogen yield. Additionally, the data achieved can be compared to existing configurations.

5.2.3. Computational fluid dynamics (CFD) analysis on the novel bioreactor configurations would enhance the understanding of these reactor configurations in regards to mass transfer, gas-liquid transfer, sheer stress and heat transfer as well the impact on cell physiology. Additionally CFD is typically done to study the dynamic behaviour of stirred systems with diverse impeller conformations.

5.2.4. Applying the novel reactor configuration in other areas of biotechnology to investigate the efficiency of the designed reactor on other fermentation processes that require biomass retention.

5.2.5. Advanced studies on this novel bioreactor configuration can be undertaken to enhance biomethane and other biofuels production on a large range of organic waste substrates.