OPTIMIZATION OF BIOHYDROGEN PRODUCTION INOCULUM DEVELOPMENT VIA HYBRID PRE-TREATMENT TECHNIQUES-SEMI PILOT SCALE PRODUCTION ASSESSMENT ON AGRO WASTE

By

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STUDENT DECLARATION

Optimization of biohydrogen production inoculum development via hybrid pretreatment techniques-semi pilot scale production assessment on agro-waste (potato peels).

I **Funmilayo Dorcas Faloye** declares that:

1. The research reported in this dissertation, except where otherwise indicated; is the result of my own findings in the Discipline of Microbiology, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg.
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Thesis Title: Optimization of biohydrogen production inoculum development via hybrid pre-treatment techniques-semi pilot scale production assessment on agro-waste (potato peels).

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

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DECLARATION 1- PLAGIARISM

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DECLARATION 2- PUBLICATIONS

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

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ABSTRACT

The challenges of energy crisis and environmental pollution are vital issues hindering the global sustainable development as a result of over dependence on fossil fuels. These are driving the need to explore renewable and environmentally friendly energy sources. Biohydrogen has emerged as an eco-friendly renewable energy source and a suitable alternative to fossil fuels. However, the commercialization of biohydrogen energy is hindered by the high production cost and low yield which necessitates novel strategies for an economically feasible production.

Some of these strategies include the development of stable inoculum, scale-up studies, and the utilization of renewable feedstock such as agro-food waste materials which are both abundant and sustainable. Inoculum pre-treatment is a vital aspect of hydrogen production technology as it contributes to the improvement of hydrogen yield. The inoculum pre-treatment method influences the community structure which in turn affects the microbial metabolism of hydrogen production.

This study investigates novel inoculum development techniques and evaluates the feasibility of biohydrogen production from agro waste (potato peels). The linear and interactive effect of these techniques on inoculum efficiency as well as the key process parameters for hydrogen production from potato peels were modelled and optimized. Further assessment of the hydrogen production dynamics at the semi-pilot scale including the microbial community structure were investigated using the 16SrRNA gene clone library sequence analysis.

A hybrid inoculum development technique of pH and Autoclave (PHA), pH and Heat shock (PHS) was modelled and optimized using the response surface methodology. The quadratic polynomial models had a coefficient of determination (R^2) of 0.93 and 0.90 and the optimized

pre-treatment conditions gave a 37.7% and 15.3% improvement on model predictions for PHA and PHS respectively. Maximum hydrogen yield of 1.19 mol H_2/ mol glucose was obtained for PHA in a semi-pilot scale process.

The interactive effect of a hybrid pH and microwave pre-treatment on mixed inoculum for biohydrogen production was investigated. The obtained model had a coefficient of determination (R^2) of 0.87. Two semi pilot scale-up processes were carried out to assess the efficiency of the developed inoculum with and without pH control on biohydrogen production. A two fold increase in glucose utilization was obtained and a molar hydrogen yield of 2.07 mol H_2/mol glucose under pH controlled fermentation compared to 1.78 mol H_2/mol glucose without pH control. Methane production was not detected which suggests the effectiveness of the combined pre-treatment to enrich hydrogen producing bacteria.

The developed inoculum was used to evaluate the feasibility of biohydrogen production from potato peels waste. The key process parameters of substrate concentration (g/L), nutrient supplementation (%), temperature (°C) and pH were modelled and optimized using the Artificial Neural Network (ANN) and Response surface methodology (RSM). The optimum conditions obtained were 50g/L of potato waste, 10% nutrients, 30°C and pH 6.5. A semi pilot production process under the optimized condition gave a hydrogen yield of 239.94mL/g TVS corresponding to a 28.5% improvement on hydrogen yield. Analysis of the microbial community structure showed the dominance of the genus *Clostridium* comprising of about 86% of the total microbial population including *C. aminovalericum, C. intestinale, C. tertium, C. sartagofome, C. beijerinckii* and *C. butyricum* in ascending order of predominance. Hydrogen consuming methanogens were not detected which further confirmed the efficiency of the hybrid inoculum pre-treatment.

This study has highlighted the development of a novel hybrid inoculum pretreatment method to establish the requisite microbial community and to safeguard the stability of biohydrogen

production. Furthermore, the potential of generating an economical feasible biohydrogen production process from potato waste was demonstrated in this work.

Keywords: Biohydrogen production, Dark fermentation, Pilot scale-up, Bioprocess modelling, Inoculum pre-treatment, Optimization.

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List of Abbreviation

ATP	Adenosine Triphosphate
BP	British petroleum
CH_4	Methane
CO_2	Carbon dioxide
COD	Chemical Oxygen demand
CFU	Colony forming unit
DNA	Deoxyribonucleic Acid
DOE	United States Department of Energy
EIA	United States Energy Information Administration
EREC	European Renewable Energy Commission
h	Hour
H_2	Hydrogen
HRT	Hydraulic Retention Time
Kg	Kilogram
mol	Mole
mins	Minutes
OLR	Organic Loading Rate
PHA	pH and Autoclave
PHS	pH and Heat shock
TS	Total Solids
TVS	Total Volatile Solids
VFAs	Volatile Fatty Acids
VS	Volatile Solids
VSS	Volatile Suspended Solids

CHAPTER 1

General introduction

1.1. The search for renewable energy sources

The year 1860 brought about global industrial revolution with the invention of the steam engine and energy sources in form of fossil fuels such as wood, coal oil and natural gas in replacement of natural human energy sources (Veziroglu and Sahin, 2008). Energy revolves around all aspects of life and plays a huge role in our daily lives; hence sustainable energy supply and environmental friendly are two crucial issues for the sustainable development of global prosperity. The demand for energy is still increasing due to overall growth in the world population as well as the growing demand for improvement in standard of living especially in developing countries; this has made the development of an efficient and sustainable energy system an imperative for sustainable socioeconomic development (Barbir and Veziroglu, 1990; UNDP, 2004).

The global energy requirement is mostly met by fossil fuels which are the primary energy source, and the sources include petroleum, coal, bitumen, natural gas and tar sand (Das and Veziroglu, 2001). The world is presently faced with the challenges of providing sufficient energy with limited fossil fuel resources, potential climate change induced by greenhouse gas emissions and insecurity by nuclear energy incompetence and the storage of radioactive materials (Barbir and Veziroglu, 1990).

Oil is the most important primary energy source consumed globally. Unfortunately, petroleum oil is in danger of depletion. According to the British petroleum (BP) annual world energy report (2014), the total proved oil reserves of the world amount to approximately 1688 billion barrels at the end of 2013 and will be sufficient to meet only 53 years of global production based on current energy consumption rates. Figure 1.1 shows the distribution of global oil reserves as at the end of 2013.

Oil resources are not sustainable with environmental, economic and geopolitical concerns as a result of their uneven distribution around the world. The Middle East as the major oil supplier currently is facing the challenges of political instability, civil unrest and terrorism which pose severe threat to the global energy security (Mecad, 2013). Also, the future of fossil fuel prices is still very unclear which makes economic growth and stability projections unreliable (Shafiee and Topal, 2009). The Middle East has 47.9% of the total world oil reserves and is the major petroleum supplier (BP, 2014) with an estimated oil capacity of 800 billion barrels. However, this oil reserves is fast declining as a result of high demand (Ruying, 2007). Soreel *et al.* (2009) reported that a peak in the oil reserve for the Middle East region is expected before 2020. As shown in Fig. 1.2, the reserve to production ratio of the Middle East which is the length of time that the remaining oil reserve would last based on the current production rate is only 78years.

The annual BP outlook report (2014) reported that the global oil production in 2013 did not meet the demand for global oil consumption which grew by 1.4 million barrels per day in 2013. Fossil fuel global consumption increased rapidly than its production despite the redundant global economic growth. The report also projected that the global energy consumption will rise by 41% from 2012 to 2035 at an average rate of 1.5% annually compare with 30% experienced over the last ten years.

Scientific reports indicated that the uncontrolled use of fossil fuels has caused disastrous effects on the global climate (Vijayaraghavan and Soom, 2006). About 98% of carbon emission results from fossil fuel combustion, therefore reduction in the use of fossil fuel to generate energy will reduce the amount of carbon dioxide and other pollutants thereby making the world a cleaner and safer place to live (Dermibas, 2008).

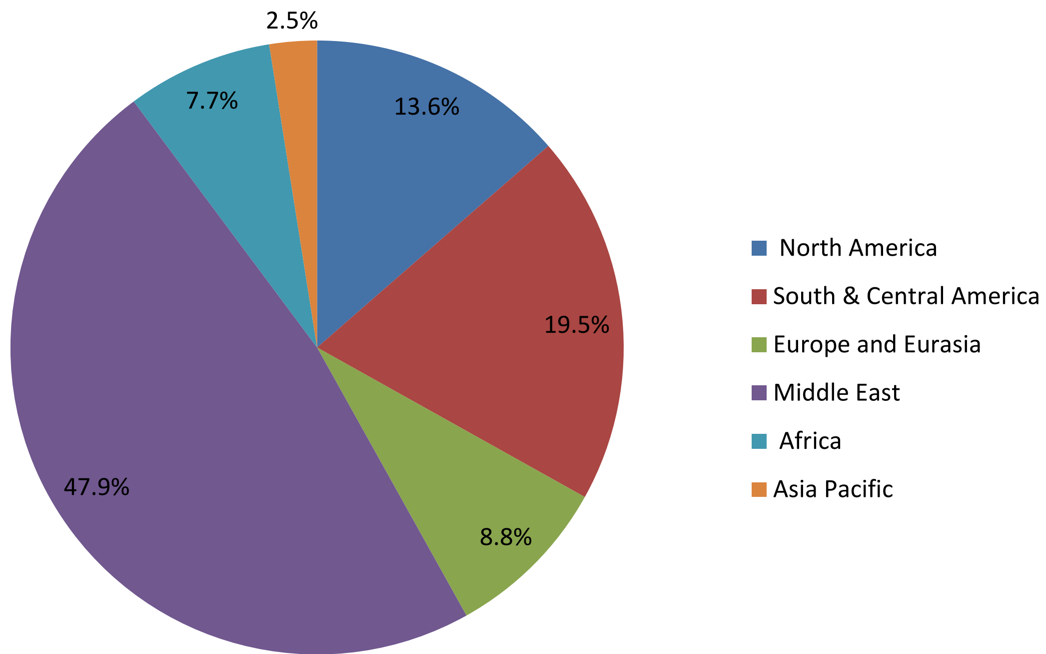

Figure 1: The distribution of global oil reserve as at 2013(BP, 2014).

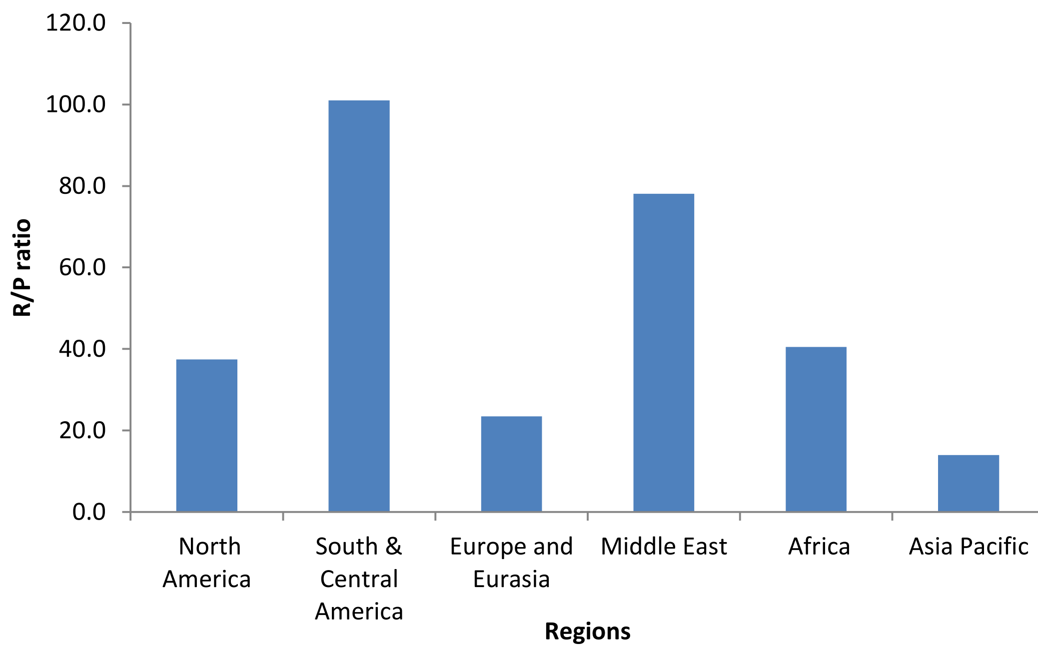

Figure 2: 2013 reserves to production ratio by region (BP, 2014).

Fossil fuel utilization can result to serious environmental and health problems as a result of carbon emission (Levin *et al*., 2004). Fossil fuel combustion causes the release of greenhouse gases, soot and ash, tar droplets and other organic compounds into the atmosphere which can lead to air pollution; oil spills and leakages can also occur during extraction, transportation and storage resulting into environmental pollution. Greenhouse gases emission has been reported to cause increase in the atmospheric temperature, a situation known as global warming and other environmental problems such as acid rain, ozone depletion, eutrophication, climate change and serious health implications (Smith *et al*., 2009; Hook and Tang, 2013). The effect of global warming ranges from increase in sea level, climate change, drought, floods, strong winds and wildfires (Barbir and Veziroglu, 1990).

Globally, it has been estimated that about three million deaths are recorded annually due to air pollution (WHO, 2008) and poor air quality from fossil fuel combustion (Pimentel *et al*., 2007). In 2012, an increase in the global carbon emission was reported with approximately 32.6 billion tons compare with 5.5 billion tons recorded in 1997(IEA, 2013). Also, reports from the annual BP Energy outlook indicated an increase in the global carbon emission in 2013 by 29% (BP, 2014). Although policies to stop carbon emission are constantly formulated, carbon emission still remain well above the tolerated level with projections that the global emission will double from 6.2 GtC in 1990 (gigatonne of carbon = 10^9 of carbon) to around 10.5 GtC by 2040 (IEA, 2011). Stern (2008) also projected that the concentration of carbon dioxide in the atmosphere could increase up to 560ppm by 2035 with a consequent rise in the atmospheric temperature above 5°C if adequate precaution is not taken.

The effect of global warming due to greenhouse gas emission cannot be ignored because of its implications on the climate, environment, economic growth and food security. The World Bank (2013) indicated that global warming could widen the poverty level as a result of its effect on food security. For instance, it is envisaged that 40% of farmlands in Sub Sahara

Africa will not be suitable for farming by 2030 due to environmental effects of heat, drought and floods which will ultimately reduce crop yields and livestock production (Cooper *et al*., 2008). Several initiatives on the reduction of carbon emission have been developed and a vital aspect will be the use of environmental friendly and sustainable source of energy.

1.2. Renewable energy

Renewable energy production has become a global priority as a result of limited fossil fuel resources, the alarming rate of environmental pollution and global warming. Renewable energy sources includes biomass, hydropower, wind, solar (thermal and photovoltaic), and marine (Ramage *et al*., 1996). The distribution of the total renewable energy consumption in the world includes biomass – 46%, hydroelectric 45%, geothermal- 6%, wind-2%, and solar-1% (Dermibas, 2008). Table 1 shows the renewable energy sources and their forms of usage.

Renewable energy is clean, inexhaustible and does not contribute to environmental pollution hence a suitable alternative to fossil fuels. Recently, there has been an increase in the search and development of clean energy generated from renewable sources, for instance renewable energy for power generation grew by approximately 16.3% in 2013 and accounted for more than 5% of the global electricity production for the first time in 2013 (BP, 2014). The European commission renewable energy roadmap sets a target to increase the share of its renewable energy to 20% of the total energy consumption by 2020 (EREC, 2006).

Renewable energy is distributed throughout the world including the under developed and developed countries. Renewable energy sources such as biomass, hydropower, wind and solar can help solve environmental and economic concerns associated with fossil fuel utilization. Renewable energy system will therefore play a pivotal role in the global energy transformation system and the world future energy supply (Domburg *et al*., 2010).

1.3. Hydrogen as a renewable energy source

Hydrogen is one of the abundant elements in the universe; it is an odourless, colourless, tasteless and non-poisonous gas (Dermibas, 2008). Hydrogen can be generated from renewable energy sources such as biomass, hydropower, solar energy using photovoltaic for direct conversion, solar, thermal energy and wind power (Miranda, 2004). Hydrogen has a great potential as an energy source with a low carbon emission and represents a cleaner and more sustainable energy system (Veziroglu, 1975). Hydrogen is storable and transferable with high heat energy per mass unit and its sources are globally distributed. Hydrogen possesses properties that make it an ideal fuel and compatible with energy technologies such as fuel cells, engines and combustion turbines (Carglar and Ozmen, 2000).

Hydrogen is considered as one of the promising fuel of the future because of its high energy efficiency, low pollution and renewable properties (Hohlein *et al*., 2000; Das and Veziroglu, 2001). Over the last two decades, hydrogen has gained global attention as an environmental friendly renewable energy source (Koroneos *et al*., 2005).

Most developed countries around the world have recognised the importance of the hydrogen economy and many researches are currently focussing on its implementation as an alternative energy source to improve energy security, economic development and environmental protection (Turner, 2004; EIA, 2011). According to the European Commission, hydrogen economy will help to provide a clean, safe and sustainable energy supply (European commission, 2003). The United States Department of Energy (DOE) projected that hydrogen energy will contribute approximately 6-10% of the total energy market by 2025 (DOE, 2004).

Hydrogen energy has numerous benefits as a universal energy carrier; it is non toxic since water is the only product when converted to energy (Midilli *et al*., 2005). Hydrogen can be produced from a wide range of energy sources although hydrogen is mostly generated from fossil fuels using electrochemical, thermochemical, photocatalytic and photo electrochemical

processes (Momirlan and Veziroglu, 2002). Globally 48% hydrogen is produced from natural gas, 30% from oil, 18% from coal and 4% from water electrolysis (Dermibas, 2008), however these processes are expensive and energy intensive (Han and Shin, 2004). Although steam reforming which is presently a commercial process of producing hydrogen is cheap, the process can lead to carbon emission during the hydrocarbon conversion (Rifkin, 2002).

Table 1: Renewable energy sources and its use (Dermibas, 2008)

Energy source	Energy conversion and usage
Biomass	Heat and power generation, pyrolysis, gasification, digestion,
Hydropower	Power generation
Geothermal	Urban heating, power generation, hydrothermal, hot dry rock
Solar	Solar home system, solar cookers and dryers
Direct solar	Photovoltaics, thermal power generation, water heaters
Wind	Power generation, wind generators, windmills and water pumps
Waves	Numerous designs
Tidal	Barrage, tidal stream

1.4. BIOHYDROGEN

Biohydrogen is defined as hydrogen produced biologically (mostly by bacteria) from waste organic materials. This includes hydrogen produced from renewable resources such as water, organic wastes or biomass either biologically or photo biologically by photosynthesis and dark fermentation process (Benemann, 1998). Biohydrogen has several benefits of low energy requirements and low cost of operation compared to photo electrochemical and thermochemical processes (Dermibas, 2008). Biological hydrogen production has become more attractive due to its ability to utilize renewable energy resources and its production at ambient temperature and pressure (Sinha and Pandey, 2011).

Biological hydrogen production technologies include a wide range of process to generate hydrogen. These are direct biophotolysis, indirect biophotolysis, photofermentation and dark fermentation (Dermibas, 2008). All these approaches are based on the ability of microorganisms to use protons (H^{+}) as an electron sink for two electron equivalents:

$$2H^{+} + 2e^{-} \longrightarrow \text{Hydrogen} \qquad (1)$$

Biohydrogen production technologies differ in the electron donor, the redox potential and the microorganisms involved in the process. A large number of microbial species including significantly different taxonomic and physiological types are able to produce hydrogen (Kotay and Das, 2008). Biohydrogen producing microorganism utilizes an array of substrates such as agricultural wastes, municipal waste, animal waste and residues (Carere *et al*., 2008; Muradov and Veziroglu, 2008). However, some of the current biohydrogen production technologies are faced with the constraints of low hydrogen yield and production rate. For instance, the scientific and technical feasibility of direct hydrogen production from sunlight is still a challenge and requires more development despite its higher theoretical energy efficiencies (Akkerman *et al*., 2002).

Biohydrogen production can be classified into 3 major categories which includes: biophotolysis of water using algae and cyanobacteria, photodecomposition of organic compounds by the photosynthetic bacteria (photofermentation) and fermentative hydrogen production from organic waste (dark fermentation) (Hallenbeck and Benemann, 2002).

The direct photolysis explores the ability of photosynthetic algae and cyanobacteria to split water directly into hydrogen and oxygen. This process takes place via the direct absorption of light and includes the transfer of electrons to the nitrogenase and hydrogenase enzymes (Manis and Baneerjee, 2002). Microorganisms under anaerobic condition are able to release the excess electrons and convert the hydrogen ions to hydrogen gas by the help of the hydrogenase enzyme (Sorensen, 2005; Turner *et al*., 2008). The green algae or cyanobacteria carries out photosynthesis and uses the captured solar energy to split water to hydrogen and oxygen through the reduction of ferredoxin. The merit of this process is the abundance of the main substrate which is water (Hankamer *et al*., 2007).

While this technology has significant promise, it is also faced with enormous challenges such as low light conversion efficiencies, the sensitivity of the hydrogenase enzyme system to oxygen, high cost of bioreactors and storage of light energy (Hallenbeck and Ghosh, 2009). The possibility of continuous hydrogen production under aerobic conditions is also a major barrier to the commercialization of this process (DOE, 2007).

Alternatively, some photosynthetic algae can produce hydrogen indirectly under certain conditions. In this photofermentation, the photosynthetic microorganisms are able to convert solar energy directly to hydrogen from organic substrates. For instance, the non sulphur purple bacteria under anaerobic condition used the captured energy to produce ATP and high energy electrons to reduce ferredoxin. This in turn will drive the proton reduction to hydrogen by the help of the nitrogenase enzyme (Hallenbeck and Ghosh, 2009; Show *et al*., 2012). The photosynthetic hydrogen production by the purple bacteria is relatively simple

compared with the green algae as these bacteria can use simple organic acids such as acetic acid and dihydrogen sulphide as electron donor (Akkerman *et al*., 2003).

The merits of this method include the complete conversion of organic wastes to hydrogen and carbondioxide and its potential for waste treatment (Hallenbeck and Ghosh, 2009). Photofermentation is still a focus of intense research in order to overcome the challenges of low light conversion rate, high energy demand and the cost implication of the photo bioreactors. Optimal process parameters such as carbon and nitrogen ratio, illumination intensity, bioreactor configuration and the inoculum age can be used to improve its yield (Basak and Das, 2007).

Fermentative hydrogen production is a more promising technology due to its high rate of hydrogen evolution, versatility of applicable substrates as well as low technical requirements compared to other biohydrogen production technologies (Kotay and Das, 2008; Nandi and Sengputa, 1998; Hawkes *et al*., 2002). This process also appears to be more favourable because of the simultaneous reduction in environmental pollutants as well as the production of clean energy (Van Ginkel and Logan, 2005; Levin *et al*., 2004). In this process, carbohydrate rich substrates are broken down by the hydrogen producing bacteria to hydrogen, organic acids (acetic, butyric) and alcohols (ethanol, butanol) under anaerobic conditions (Hallenbeck, 2009).

1.5. Research Motivation

Hydrogen based economy will contribute to the mitigation of greenhouse gas emissions and help build a clean and sustainable energy system. Despite the current intensive research on fermentative biohydrogen production, its commercialization is still faced with the major challenges of high production cost and low yield thus there is an urgent need to come up with strategies that could make it more economically feasible.

Improvement in biohydrogen production could be achieved through process optimization whereby process parameters such as temperature, pH, substrate concentration, hydraulic retention time (HRT) which plays crucial role in the production process as well as the activity of the hydrogen producing bacteria. The importance of process optimization cannot be overlooked in any bioprocess as the control of the process parameters at the optimal value could make the process to achieve maximum productivity with the lowest process cost.

Furthermore, one of the ways to lower the production cost is the use of cheap and renewable feedstock such as agricultural and food residues which are both abundant and sustainable. Recent studies on the assessment of bio energy potential in Africa indicated approximately 10PJ/yr to 5254PJ/yr (PJ-Pentajoule: 10^{15} Joule) for crop residues and waste in Africa available for energy production by 2020 (Stecher *et al*., 2013). With this large volume of agro-food waste, Africa thus possesses significant potential for producing biohydrogen from agro-food wastes.

Fermentative hydrogen production can be performed by pure and mixed microbial cultures; however, the use of pure cultures in dark fermentation is potentially more expensive than employing mixed cultures as it requires sterile conditions and strict process control especially for industrial production. On the other hand, the use of mixed cultures is prone to producing lower hydrogen yields due to the presence of hydrogen consuming methanogens and homoacetogens alongside the competition for substrates by the non-hydrogen producing microbial populations. Inoculum development is one of the most important aspects of the fermentative biohydrogen process with the key role of selecting the requisite microorganisms for enhanced hydrogen production and conversion efficiency of substrate.

Fermentative hydrogen production is controlled by several factors such as inoculum composition, substrates, inorganic nutrients and operating conditions, therefore an appropriate design to optimize these factors will help to improve productivity and yield hence the basis of this research.

1.6. Aims

The research aims at enhancing biohydrogen production through inoculum development and optimization of the operational parameters on agricultural residues for industrial scale up.

The specific objectives are:

- Modelling and optimization of anaerobic sludge inoculum development for hydrogen production on combinations of pre-treatment techniques.
- Evaluation of the potential of potato peel waste for biohydrogen production
- Modelling and optimization of hydrogen production from potato peel waste using the Response Surface methodology and Artificial Neural Network.
- Preliminary assessment of biohydrogen production process dynamics from potato peels waste at a semi pilot scale.
- Analysis of microbial community structure during hydrogen production

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CHAPTER 2

Literature Review

2. Fermentative Biohydrogen production

Fermentative hydrogen production is the fermentative conversion of organic substrate to biohydrogen by a diverse group of bacteria using a multienzyme system involving three steps similar to anaerobic digestion. Fermentative or hydrolytic bacteria hydrolyze complex organic polymers to monomers which are further converted to a mixture of lower molecular weight organic acids and alcohols by the hydrogen producing acidogenic bacteria. The production of biohydrogen occurs in the second and third step; therefore methanogenic bacteria must be inhibited to avoid the consumption of hydrogen to produce methane (Dermibas, 2008). Dark fermentation for hydrogen production from wastes could be a better alternative to fossil fuel derived hydrogen providing a practical approach for biohydrogen production (Li and Fang, 2007).

Fermentative hydrogen production occurs in anoxic conditions, the electrons which need to be disposed off to maintain electrical neutrality are produced and the protons act as the electron acceptor for hydrogen production with the aid of hydrogenase enzyme (Sinha and Pandey, 2011). The fermentative biohydrogen production depends on the ability of the hydrogen producing bacteria to use proton as an electron sink during the fermentation of organic substrates. In other words, the process involves the utilization of pyruvate-ferredoxin hydrogenase or pyruvate formate lyase and an organic compound as the electron donor (Lee *et al*., 2009).

Carbohydrates are the preferred organic carbon source for hydrogen producing fermentations; glucose fermentation will yield 2mol or 4mol of hydrogen per mole with butyrate and acetic

acid as their respective fermentation product. Dark fermentative biohydrogen production has the advantage of high hydrogen yield and production rate, low cost of operation and the utilization of various organic waste substrates and wastewater(Hallenbeck and Ghosh, 2009; Sinha and Pandey, 2011).

Currently, fermentative biohydrogen production is undergoing intensive research and development at laboratory scale with focus on production from biomass such as agricultural wastes, municipal waste, and industrial wastewater using anaerobic hydrogen producing bacteria. The production reactor system can be operated in either batch or continuous mode. Several authors have reported fermentative hydrogen production in a batch mode which is generally more appropriate for research purpose, however reports on a semi-pilot scale or pilot scale continuous production for practical application are still limited.

Recently, Lin *et al*. (2010) reported a high rate hydrogen production system in a 400L pilot scale system with a maximum hydrogen production rate of 15L/L/h. The reactor was operated in a continuous mode for one month and fed with 20g COD/L at 35°C. Also, a maximum hydrogen fraction of 37% corresponding to a hydrogen yield of 1.04mol H_2/mol sucrose and hydrogen production rate of 15.59m^3/m^3/d was reported in a pilot scale fermentor operated for 67days at 35°C. The seed inoculum was granular sludge fed with 20-40kg COD/m^3 (Lin *et al*., 2011).

2.2 Dark Fermentation Pathway

Dark fermentation is a process that occurs under anaerobic conditions. During fermentation, hydrogen producing bacteria use the reduction of protons to produce hydrogen via the hydrogenase. This is required to maintain electrical neutrality for continuous supply of ATP generated by substrate level phosphorylation (Adams *et al.*, 1980). Hydrogen can be produced from various substrates, although it is of great advantage to produce hydrogen from

organic wastes by fermentative technology as this method not only treat the organic waste but also produces clean energy (Sinha and Pandey, 2011).

Hydrogen production is generally achieved by 2 methods with the help of specific co-enzyme and each hydrogen production pathway is related with the specific bacteria that possess such features required for optimum hydrogen production. The two methods are:

a) Reoxidation of NADH pathway

b) Formic acid decomposition pathway

The production of hydrogen via the glycolytic pathway is common in *Clostridium* species in which hydrogen is produced under the acidogenic conditions catalysed by the ferredoxin oxidoreductase and hydrogenase (Tanisho *et al*., 1998). During fermentation, glucose is first converted to pyruvate to produce the reduced form of nicotinamide adenine dinucleotide (NADH) via the glycolytic pathway. Pyruvate is then converted to acetyl coenzyme A (acetyl CoA), carbon dioxide and hydrogen by pyruvate ferredoxin oxidoreductase and hydrogenase. During the decarboxylation of pyruvate to acetyl CoA, electrons will move to ferredoxin resulting in the production of proton and the release of hydrogen (Thauer *et al*., 1997; Saint-Amans *et al*., 2001).

Pyruvate can also be converted to acetyl CoA and formate which is readily converted to carbon dioxide and hydrogen. Acetyl CoA is finally converted to some soluble metabolites such as acetate, butyrate and ethanol (Hawkes *et al*., 2007; Li and Fang, 2007).

NADH- ferredoxin reductase functions primarily as an electron carrier and is involved in pyruvate oxidation to acetyl CoA and carbon dioxide as well as proton reduction to hydrogen The disposal of electrons via pyruvate-ferredoxin oxidoreductase or NADH- ferredoxin oxidoreductase and hydrogenase might be affected by the corresponding NADH and acetyl CoA levels as well as environmental conditions, therefore the oxidation- reduction state must

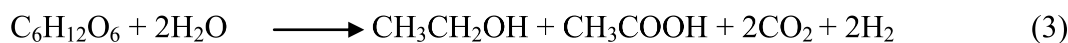
be balanced through the NADH consumption to form some reduced compounds such as lactate, ethanol and butanol resulting in a lowered hydrogen yield (Lee *et al*., 2011).

Acetate and butyrate are the two common products of carbohydrate fermentation. Theoretically, 4 mole of hydrogen can be produced from 1 mole of glucose via the acetate pathway resulting in the production of acetic acid (Equ.1) and 2 mole of hydrogen from 1 mole of glucose via the butyrate pathway to produce butyrate (Equ. 2) (Nandi and Sengputa, 1998; Hawkes *et al*., 2007).

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

$$C_6H_{12}O_6 \longrightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2)$$

In addition, ethanol can be produced with the release of 2 mole of hydrogen per mol of glucose as shown in Equation 3 (Hwang et al., 2003; 2004).



$$C_6H_{12}O_6 + 2H_2O \longrightarrow CH_3CH_2OH + CH_3COOH + 2CO_2 + 2H_2 \quad (3)$$

Clostridium species are the dominant hydrogen producing bacteria and usually employs the acetate/butyrate fermentation pathway (Fang *et al*., 2002; Van Ginkel and Logan, 2005a, b; Lee *et al*., 2008; Lee and Rittmann, 2009). *Clostridium* species produces hydrogen gas during the exponential growth phase, during the stationary phase its metabolism will shift from hydrogen or acid production to solvent production and other by products. The hydrogen produced from glucose is often determined by the acetate to butyrate ratio produced during fermentation (Han and Shin, 2004). The pathway for glucose fermentation is illustrated in Figure 2.1.

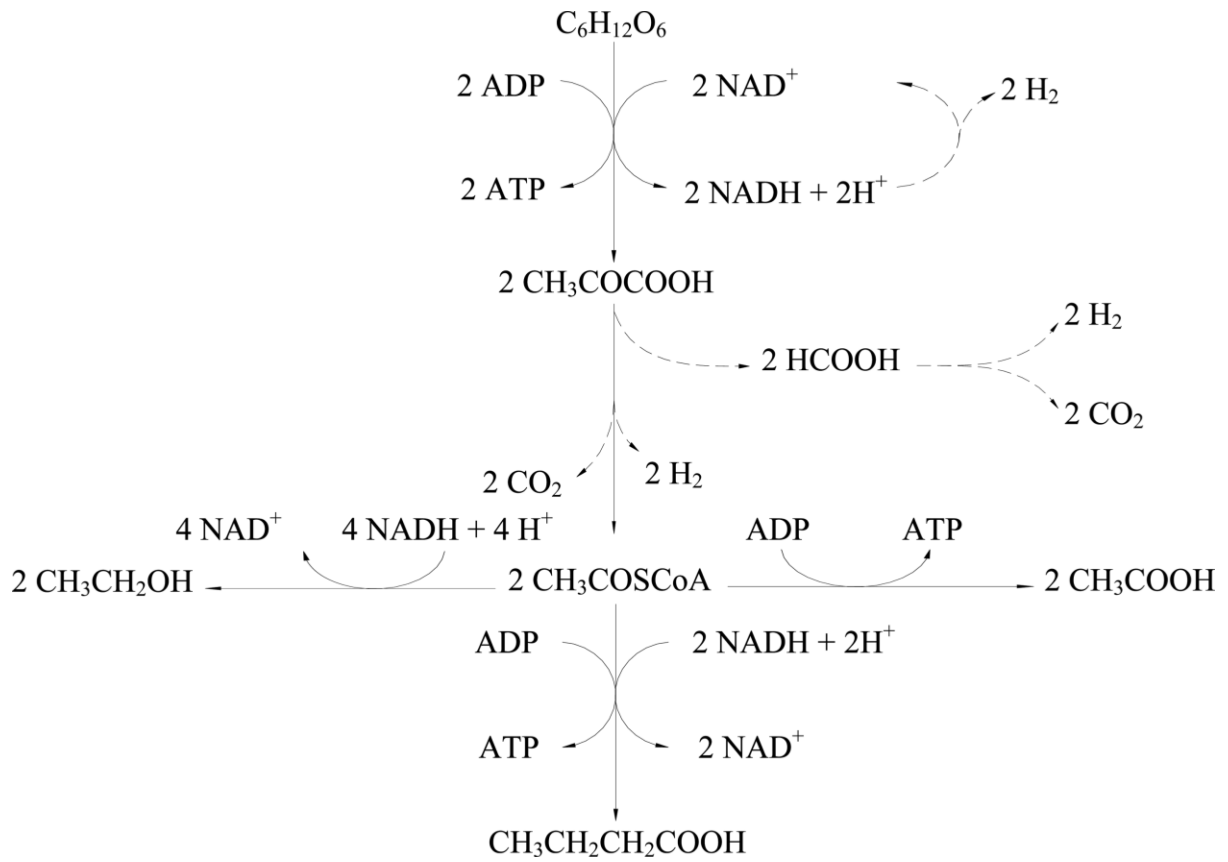


Figure 1: Fermentative hydrogen production glucose pathway (Tanisho, 2001; Ren and Wang, 2004).

The facultative anaerobes belonging to the family of *Enterobacteriaceae* (*Klebsiella sp., Escherichia coli, and Enterobacter sp.*) can metabolize pyruvate to formic acid in a process known as Formic acid fermentation. The process is initiated by the conversion of pyruvate to formic acid by pyruvate formate lyase resulting in the production of acetyl-CoA and conservation of energy by the formation of ATP via acetyl phosphate. The formic acid can further be broken down into hydrogen and carbon dioxide via the formic hydrogen lyase under anaerobic conditions and suitable electron acceptors (Equation 4a and 4b) (Nakashimada *et al.*, 2002; Shin *et al.*, 2007).

$$CH_3COOH + HCOA \longrightarrow CH_3COCOA + HCOOH \qquad (4a)$$

$$HCOOH \longrightarrow H_2 + CO_2 \qquad (4b)$$

The NADH produced by the bacteria of the genus *Enterobacter* is rarely used to produce hydrogen due to the lack of specific co-enzymes such as ferredoxin oxidoreductase. Some microorganisms are able to unravel this problem through the inhibition of the pyruvate dehydrogenase using pyruvate or its derivatives as an electron acceptor for the reoxidation of NADH during fermentation.

Formic acid fermentation is divided into two types: The mixed acid and butanediol fermentation. Mixed acid fermentation is characterised by the production of ethanol and mixture of organic acids such as acetic, lactic, succinic acid as observed in *Escherichia coli*, *Salmonella*, *Proteus*. Butanediol fermentation involves the conversion of pyruvate to acetoin which is finally reduced to 2-3 butanediol, NADH, ethanol and low amounts of mixed acids. The production of these reduced compounds normally have an adverse effect on hydrogen production resulting in a low yield lesser than 2 mol-H_2/ mol glucose. This process is commonly observed in species such as *Enterobacter*, *Serratia*, *Erwinia* and *Bacillus* (Lee *et al.*, 2011).

It is noteworthy to mention that the biochemical reaction is slightly different in the thermophilic hydrogen producing bacteria. The breakdown of glucose is achieved through the Embder-Meyerhof pathway as observed in eubacterium *Thermotoga maritama* or through the modified Embder-Meyerhof pathway in *Thermococales*. In the modified Embder-Meyerhof pathway, the fermentation is not coupled with ATP synthesis rather the conservation of energy is accomplished through the formation of acetate from acetyl-CoA synthetase. Apart from acetate, alanine which is a reduced end product of L-alanine is produced with the aid of alanine aminotransferase through the process of transamination with glutamate (Kanai *et al*., 2005). Hydrogen and alanine produced are both used for the disposal of intracellular reducing products, therefore inhibiting the formation of alanine may enhance the release of hydrogen gas (Lee *et al*., 2011). Schroder *et al*. (1994) reported that the re-oxidation of NADH typical of the *Clostridia* is also used by the hyperthermophilic eubacterium for the production of hydrogen.

Quite a number of bacteria species with vast taxonomic and physiological characteristics can produce hydrogen via biochemical reactions. Hydrogen producing microbes use hydrogenase and or nitrogenase enzyme as the hydrogen yielding protein. Both enzymes regulate the hydrogen metabolism of many prokaryotes and some eukaryotic organisms including green algae (Hallenbeck and Benemann 2002; Lee *et al*., 2009).

2.3. Microbiology of Biohydrogen Production

Fermentative hydrogen production is an integral part of the process of anaerobic digestion which involves the interaction of different microorganisms. Anaerobic digestion occurs in four phases and the first two phases are very vital for biohydrogen production.

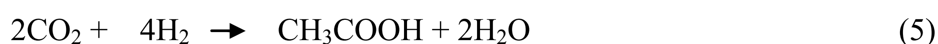
Hydrolysis is the first step in which complex organic polymers are broken down into simpler compounds. This process is catalysed by an array of extracellular hydrolytic enzymes such as

cellulose, amylase and protease. The end products of this phase are used up by the bacteria for metabolism (Gavrilescu, 2002).

Acidogenesis is the second phase in which the soluble monomers are used as source of carbon and energy by the fermentative hydrogen producing bacteria to produce volatile fatty acids, alcohols and biogas (Reith *et al*., 2003). This phase is very crucial and process conditions such as pH, temperature, substrate concentration must be optimized such that the process can be directed towards the generation of desired products (Masilela, 2011). Hydrogen is a key intermediate which can be rapidly consumed by other microorganism in the mixed culture such as homoacetogens, methanogens and sulphate reducing bacteria if not inhibited(Das and Veziroglu, 2008; Valdez-Vasques and Poggi-Varaldo, 2009; Guo *et al*., 2010).

Acetogenesis phase is characterised by the reduction of end products of acidogenesis such as aromatic compounds, volatile fatty acids (VFAs) and alcohols which are converted to acetic acid and hydrogen. Acetate, butyrate and propionate are the major intermediate products in this phase. Acetic acid as end product of the fermentation can result to a theoretical production of 4mol of hydrogen per mole of hexose which is equivalent to 498ml H_2 per gram of hexose (0°C, 1atm) for acetic fermentation; while in butyrate pathway a lower molar hydrogen yield of 2mol of hydrogen per mole of hexose is observed which is equivalent to 249ml H_2 per gram of hexose (0°C, 1atm) (Hawkes *et al*., 2007).

However, the accumulation of acetate during the fermentation does not imply higher biohydrogen production since many microbial species can convert hydrogen and carbon dioxide to acetate (Equ.5) (Antonopoulos *et al*., 2008)



$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \quad (5)$$

Propionate, ethanol, and lactic acid may also accumulate during fermentation with mixed culture and a ratio of 3:2 of butyrate / acetate is usually observed resulting in a theoretical

average yield of 2.5 mole of hydrogen per mole of hexose (Hawkes *et al*., 2007). Propionate is a metabolite of a hydrogen-consuming pathway, while ethanol and lactic acid are involved in a zero hydrogen balance pathway (Guo *et al*., 2010).

Methanogenesis is the conversion of hydrogen, acetate and carbon dioxide produced in the acetogenic phase to methane (CH_4) by the methanogenic bacteria. Aceticlastic methanogens and hydrogenotrophic methanogens are the two groups of bacteria involve in this phase as shown in Equations 6 and 7 respectively.

$$\text{Acetate} + H_2O \rightarrow CH_4 + HCO_3 \quad (6)$$

$$4H_2 + HCO_3 + H \rightarrow CH_4 + 2H_2O \quad (7)$$

2.4. Biohydrogen Producing Bacteria

Diverse group of microorganism are capable of producing hydrogen through the process of dark fermentation. In general, hydrogen producing bacteria belonging to the genera *Enterobacter* and *Clostridium* have been widely studied (Collet *et al*., 2004; Kumar and Das, 2000). *Enterobacter* spp are gram negative, rod shape and facultative anaerobes, they metabolise glucose through the mixed acid or 2, 3 butanediol fermentation (Nath and Das, 2004). Perego *et al*. (1998) reported a hydrogen yield within the range of 0.2-1.8mol H_2/mol glucose by *E. coli* NCIMB 11943 using glucose or starch hydrolysate. Also an increase in hydrogen yield was reported by Kumar *et al*. (2001), this improvement was achieved through the blocking of organic acid formation pathway using the proton suicide technique on *E. aerogenes* and *E. cloacae*. A higher hydrogen yield of 3.1 mol H_2/mol glucose was achieved by using hyd over expressed recombinant gene from *E. cloacae* in a non hydrogen producing *E. coli* (Chittibabu *et al*., 2005). Research has focussed more on the use of pure cultures of the genus *Enterobacter* because they are easier to grow compare with the obligate anaerobes.

The hydrogen producing ability of the genus *Clostridium* has been well reported including *Clostridium acetobutylicum* (Zhang *et al*., 2006a; b), *C. butyricum* (Chen *et al*., 2005), *C. pasterianum* (Meyer and Gagnan, 1991), *C. paraputrificum* (Evvyernie *et al*., 2001), *C. lentocellum* (Ravinder *et al*., 2000), *C. thermosuccinogenes* and *C. bifermentans* (Wang *et al*., 2003b), *C. thermolacticum* (Collet *et al*., 2004). They are strict anaerobes and the dominant hydrogen producing bacteria using the acetate/ butyrate fermentation pathway (Li and Fang, 2007). Optimum hydrogen yield varying between 1.1 mol H_2/ mol glucose and 2.6 mol H_2/ mol glucose have been reported for *Clostridium* spp based on substrate type as well as the process conditions. In addition, aerobic bacteria such as *Bacillus* (Kalia *et al*., 1994; Shin *et al*., 2004) *Aeromonas* spp, *Pseudomonas* spp and *Vibrio* spp (Oh *et al*., 2003) have been implicated in fermentative hydrogen production although with a yield lesser than 1.2mol-H_2/ mol glucose.

Anaerobic thermophilic microbes belonging to the genus *Thermoanaerobacterium* have also been investigated for their ability to produce hydrogen during dark fermentation. They were found to be the dominant microbe in the thermophilic hydrogen fermentation (Zhang *et al*., 2003). Several species of *Thermoanaerobacterium* including *T. thermosaccharolyticum*, *T. polysaccharolyticum*, *T. zeae*, *T. lactoethylicum*, *T. aotetoense* were reported for their hydrogen producing ability. These thermophilic bacteria appear to possess a greater ability to produce hydrogen up to 4.0 mol-H_2/mol glucose (Schroder *et al*., 1994). Collet *et al.* (2004) achieved a hydrogen yield of 1.5 mol-H_2/ mol glucose using *C. thermolaticum*.

Other thermophiles reported include *Klebsiella oxytoga* HP1 with a maximum hydrogen yield of 3.6 mol-H_2/ mol glucose at 38°C. Hyperthermophiles are known to demonstrate very low hydrogen production rate ranging from 0.01- 0.02H_2/L-h compared with the mesophiles which may be due to the lower growth rate and glucose utilization observed during fermentation (Lee *et al*., 2011).

2.4.1. The use of pure or mixed cultures

Pure cultures have been explored in fermentative hydrogen production with various substrates ranging from simple sugars to complex organic waste. Although pure cultures have been shown to produce hydrogen, however they have limited applications especially for industrial biohydrogen production due to the difficulty of maintaining sterility and preventing contamination (Wang *et al*., 2003a).

Hydrogen producing microbes are also present in natural microenvironment such as soil, sewage sludge, compost, wastewater, animal dung (Sinha and Pandey, 2011). Research focussing on the use of mixed microflora has been well reported with simple sugars as well as organic waste (Nandi and Sengputa, 1998; Das and Veziroglu, 2001; Levin *et al*., 2004; Kotay and Das, 2008). Mixed culture utilization offers an overall improvement in biohydrogen production efficiency especially for industrial applications due to its requirement for minimal sterilization, presence of high microbial diversity resulting into improved substrate conversion and increase in adaptation capability, possibility for mixed substrates co fermentation, higher capacity for continuous biohydrogen production system and applicability of wider source of substrate (Temudo *et al*., 2007; Omhici and Kargi, 2010; Wang *et al*., 2010; Abreu *et al*., 2012; Lay *et al.*, 2012).

Microbial community studies of the mixed microflora revealed the dominance of the genus *Clostridium* and their hydrogen producing capability (Fang and Liu, 2002; Hung *et al*., 2007; Wang and Wan, 2008) with over 60% of the total microbial population after pre-treatment (Fang *et al*., 2006; Wang *et al*., 2008a).

However, in a mixed culture system the hydrogen produced is readily consumed by the hydrogen consuming bacteria since both coexist together in the mixed culture, thus in order to exploit hydrogen production from a mixed culture system, the mixed inoculum must be subjected to pre-treatment to inhibit the activity of the hydrogen consuming bacteria at the

same time enriching the hydrogen producing bacteria. This strategy is based on the spore forming nature of the hydrogen producing *Clostridium* which is commonly found in sludge, compost, soil and sediment (Brock *et al*., 1994).

2.5. Agricultural wastes as substrates for biohydrogen production

Fermentative hydrogen production has received substantial interest compared to other physical and chemical methods with the advantages of process simplicity, better process economy resulting from lower energy requirements, higher production rates and utilization of low value wastes as substrates (Levin *et al*., 2004; Kim and Dale, 2004). In order for these benefits to be harnessed, feedstock for bio hydrogen production must meet specific requirements including the carbohydrate content, sustainability of the resources, its conversion and recovery should require less energy input and low cost (Hawkes *et al*., 2002). Many studies investigating hydrogen production by dark fermentation have used simple sugars such as glucose or sucrose as model substrates and very few have looked into solid substrates conversion. For organic materials to be potentially useful as substrates for sustainable hydrogen production they must not only be abundant and readily available but also cheap and highly biodegradable (Guo *et al*., 2010). Hydrogen must be produced from renewable raw materials for its production to be sustainable, feedstock such as agro residues and lignocellulosic products such as wood and wood waste when utilised effectively can become a major source of renewable hydrogen. Also, several processes that produce hydrogen from biomass are complementary to those that produce bio products; therefore countries with great agricultural economies can effectively improve their economy through the incorporation of renewable energy production into agro industry.

Agricultural residues and food waste are the most abundant, cheapest and most readily available organic waste to be biologically transformed; they include straw, stover, peelings, cobs, stalks, bagasse, and other lignocellulosic residues (Mtui, 2008). Agro residues such as

leaves, grass and crop stalks are abundant in the world with approximately annual global yield of 220 billion tons (Khamtib *et al*., 2011). They are the cheapest and most readily available organic waste to be biologically transformed and to varying degrees can be converted biologically in anaerobic digestion processes to biohydrogen because of their biodegradability (Saratale *et al*., 2008).

Reports from the literature summarized the composition of different crop residues, for example wheat straw, corn stover and rice straw contain cellulose, hemicelluloses and lignin in a range of approximately 32- 47%, 19- 27% and 5- 24% respectively (Saratale *et al*., 2008) with a reasonable hypothesis that biohydrogen yields may be indirectly related to the cellulose and lignin contents of the feedstock (Buffiere *et al*., 2006). Availability, carbohydrate content, fermentability and cost of the raw materials are important factors to be considered for large scale hydrogen gas production. Cellulose and starch containing renewable resources (biomass) and wastes are the most suitable raw materials for biohydrogen production as a result of high carbohydrate content, availability and low cost (Sagnak *et al*., 2011).

Variety of agro residues such as barley, wheat straw, corn straw, corn stalk, corn stover, rice straw, sugar beet, ground wheat, sugarcane bagasse, leaves have been used as feedstock for biohydrogen production through dark fermentation. Utilization of starch containing agro residues for biohydrogen production have special advantages over other raw materials due to high carbohydrate, nutrient(N, P) and minerals (Fe, Zn, Mg) contents (Zhu *et al.,*2005).

Corn cob is an ideal raw material for producing hydrogen. It is an abundant agricultural by product with low commercial value which makes it an important feedstock in biohydrogen production (Pan *et al*., 2010). Currently, the effective utilization of corn cob for biological processing to obtain end products with added value is of interest worldwide (Yang *et al*., 2006). A successful bioconversion of biomass to hydrogen depends strongly on the pre-

treatment of raw materials to produce feedstock which can be fermented by the hydrogen producing bacteria.

Pan *et al*. (2010) investigated the production of biohydrogen from corn cob using natural anaerobic microflora. The optimum pre-treatment condition for corn cob was reported to be 100˚C, 30 ns nd 1% HC ( ) 107 9 TVS and 420m/ g TVSh^{-1} were reported as the maximum hydrogen yield and hydrogen production rate respectively. Hydrogen yield from the pre-treated corn cob was much higher than the raw corn cob; hence the acid pre-treatment played a crucial role on the hydrogen production. Optimum substrate concentration of 5g/l was reported which contributed to the shortened lag phase period and the quick adaptation of hydrogen producing microorganisms with a significant increase in the cumulative hydrogen yield.

Corn stalk is the stalk or stem of a corn plant. The main composition of the raw corn stalk wastes includes moisture (8.96%), hemicelluloses (20.87%), cellulose (38.92%), lignin (21.52%), protein (2.74%), and unidentified materials (6.99%) (Yang *et al*., 2006). Biohydrogen production from the corn stalk wastes with acidification pre-treatment in a batch fermentation test was reported by Zhang *et al*. (2007a) and the influence of several environmental factors on biohydrogen production. The maximum cumulative hydrogen yield of 149.69 ml-H_2/g TVS was reported at initial pH 7.0 and substrate concentration of 15g/l pre-treated with 0.2% HCl. Acetate, propionate, butyrate and ethanol were the main metabolic by-products during the conversion of cornstalk into hydrogen.

Biohydrogen production from cornstalk was optimized using the response surface methodology based on a three factor-five level central composite designs (CCD). The maximum hydrogen yield of 205.5ml-H_2/g TVS was reported at 7.5 days, 46 3˚C nd 0.049g/g for HRT, temperature and substrate concentration respectively. The high biohydrogen production potential from cornstalk is attributed to soluble sugar by solid state

enzymolysis resulting to direct biodegradation of the corn stalk during the fermentation process (Xing *et al*., 2011). Datar *et al*. (2007) investigated the production of hydrogen using corn stover inoculated with a microbial consortium obtained from a local wastewater plant. The study confirmed that in addition to glucose, lignocellulosic biomass also contains an array of five carbon sugars such as xylose and arabinose.

Rice straw is one of the largest lignocellulosic agro residues produced in the world, especially in many Asian countries (666.7 million tons in annual supply) (Kim and Dale, 2004). Every year, more than 113 million tons of rice hulls are generated throughout the world (Yu *et al*., 2009) which is managed by burning or burying on site resulting into significant environmental pollution (Laura and Porcar, 2009). The use of rice straw and hulls to produce biohydrogen is a promising and effective method of managing this waste and at the same time producing environmental friendly energy. Generally, rice straw consists of lignin (5- 24 %), hemicelluloses (19-27%), cellulose (32- 47%) and inorganic components (5-10%), the hemicellulose and pentose is easily hydrolyzed and released. Theoretically, 41.0%-43.4% of glucose, 14.8- 20.2% xylose, 2.7- 4.5% of arabinose, 1.8% of mannose and 0.4% of galactose can be obtained in the rice straw hydrolysis (Garrote *et al*., 2002; Saha, 2003).

The cellulose in rice straw is tightly surrounded by hemicellulose and lignin which makes it difficult to hydrolyze the straw directly into fermentable sugars which will be utilized by microorganisms for biohydrogen production. Therefore efficient pretreatment method is essential to ensure maximum productivity. Several researchers have investigated fermentative thermophilic hydrogen fermentation with combined pre-treatment method of ammonia and dilute sulphuric acid, a maximum hydrogen yield of 2.7mmol/H_2/g straw was achieved (Nguyen *et al*., 2010).

Wheat waste constitutes another renewable resource that can be used as feedstock for biohydrogen because of its high starch and gluten content (> 90%) (Sagnak *et al*., 2011). In

many countries including the USA, wheat straw is an abundant by product of wheat production. The average yield of wheat straw is 1.3-1.4kg/kg of wheat grain (Montane *et al*., 1998). Wheat straw is produced throughout the world as a by-product of wheat cultivation. In developing countries like China, some of the wheat straw is used as animal feed, feedstock for paper industry and organic fertilizer, however large amounts are burnt or discarded as environmental pollutants (Ren *et al*., 2007). Wheat straw is a suitable raw material for biohydrogen production because of its high cellulose content (Esmaeilzadeh *et al*., 2010). Simultaneous saccharification and fermentation proved to be the most effective and economical way of converting wheat straw to biohydrogen with a shorter lag phase for gas production (Nasirian *et al*., 2011). Sagnak *et al*. (2011) reported the maximum hydrogen yield of 1.46mol-H_2/ mol glucose with initial sugar concentration of 10g/l from ground wheat subjected to acid hydrolysis (pH-3 0) 90˚C 15 nu s us n n u c

Sugar cane is one of the most important industrial crops. Sugarcane bagasse is a waste left after sugar cane extraction process accounting for approximately 25% of sugar cane mass and about 12 million tons annual production (Almazan *et al*., 2001). The most common use of sugar cane bagasse is energy production by combustion which can cause environmental problem as a result of CO_2 emission (Neureiter *et al*., 2002). Sugarcane bagasse consists of 3 main fractions which are cellulose, hemicellulose and lignin which can be liberated by hydrolysis and subsequently fermented by microorganisms to form different by products (Gamez *et al*., 2005). Dilute acid treatment of the hemicelluloses fraction in sugarcane bagasse yields mainly glucose and xylose with small amount of arabinose (Aguilar et al., 2002). Due to its composition, hydrolysis of sugarcane bagasse is a suitable feedstock for biohydrogen production. The use of sugarcane bagasse as renewable energy source has been the focus of study in many countries of the world with regard to development of renewable energy, the report by Dermirbas and Balat (2006) highlighted the energy potential of bagasse.

Several authors have reported the use of sugar cane bagasse and straw as feed stock to produce biohydrogen (Taniso and Ishiwata, 1994; Pattra *et al*., 2008).

Apple pomace accounts for about 25% of fresh apple fruit weight which is mainly composed of pericarp, core and pulp remains. In China alone, the yield of apple pomace as a by-product of juice extraction is more than one million tons, but only a small amount of apple pomace is processed and its disposal as waste results into serious environmental pollution and a huge loss of resources. Furthermore, apple pomace is highly biodegradable and rich in sugars, fibres, vitamin C and minerals hence a potential substrate for hydrogen production fermentation (Joshi *et al*., 1996). Feng *et al*. (2010) investigated the production of biohydrogen from apple pomace by fermentation using natural mixed microbes in a batch system and different environmental conditions. They reported the optimal pretreatment of soaking in 6% ammonia liquor for 24hrs at room temperature and initial pH 7.0 and 15g/l substrate. A maximum hydrogen yield of 101.08/gTS and hydrogen production rate of 8.08ml/g TS/h was achieved.

Other agro residues that have been investigated as feedstock for biohydrogen production includes carrot pulp which is a vegetable residue that is formed as a co product in the production of carrot juice (devrije *et al*., 2010), Oat straw (Arriaga *et al*., 2011), Sweet sorghum extract (Antonopoulos *et al*., 2008), rotten dates (Abd-alla *et al*., 2011), Grass (Cui and Shen, 2012), Poplar leaves (Cui *et al*., 2010) and mushroom waste (Li *et al*., 2011).

2.6. Process Parameters affecting fermentative biohydrogen production

Fermentative hydrogen production is a complex process that is influenced by several factors which includes inoculum type, substrate, reactor configuration, and temperature, pH and metal ions.

Inoculum Type

The use of mixed cultures in dark fermentation may be more feasible than pure cultures because of simplicity in terms of operation and control, as well as the applicability of broader source of substrates (Li and Fang, 2007). However, the use of mixed culture is faced with the challenges of co existence of both the hydrogen producing and hydrogen consuming bacteria. This necessitates the need to pretreat the mixed culture inoculum prior to fermentation in order to suppress the activity of the hydrogen consuming bacteria at the same time enriching the hydrogen producing bacteria.

Several techniques have been used to pretreat mixed culture inoculum for fermentative hydrogen production. Such methods include heat shock, alkaline, freezing and thawing, aeration, acid, microwave, sonication and methanogenesis inhibitors (such as chloroform) (Cai *et al*., 2004; Wang and Wan, 2008a; Ren *et al*., 2008; Guo *et al*., 2008; O-Thong *et al*., 2009; Luo *et al*., 2010a; Wang *et al*., 2011; Song *et al*., 2012). There exists a lot of controversy regarding the best pre-treatment method; this may be attributed to variation in terms of the source of inoculum, substrate type, and other process conditions.

Heat shock treatment has been widely used, although some authors believed that the treatment may not be effective. For instance, Oh *et al*. (2003) reported the inefficiency of heat shock pre-treatment to inhibit some homoacetogenic hydrogen consuming bacteria resulting in a decrease in hydrogen production. The activity of some hydrogen producing bacteria particularly the non endospore forming *Enterobacter* may also be affected during heat shock pre-treatment (Wang and Wan, 2008a).

Substrate type

Different kinds of substrates have been utilized in fermentative hydrogen production with both pure and mixed cultures. Generally, simple sugars such as glucose, sucrose have been widely reported, however in the recent time research is focussed on the use of complex

organic waste and industrial waste water which is abundant and cheap. Substrate type can influence greatly the hydrogen producing bacteria metabolic pathway particularly hydrogen evolution and the distribution of other by products (Wang and wan, 2009c). For instance 1, 3 propanediol and low hydrogen yield was achieved in a fermentative hydrogen production with *Clostridium butyricum* VP13266 fed with a glucose-glycerol mixture. However, when the bacterium was fed with glucose only, an improvement in the hydrogen yield was achieved (Saint-Amans *et al*., 2001).

Reports from literature also confirmed that increase in substrate concentration could lead to an improvement in hydrogen yield by the hydrogen producing bacteria although beyond certain limit increase in substrate concentration may cause a deleterious effect on hydrogen yield and production rate(Van Ginkel *et al*., 2001; Lo *et al*., 2008). Some complex substrates such as lignocellulosic agro residues are abundant and attractive for hydrogen production due to their carbohydrate content. However such substrates need to be pre-treated in order for the sugar to be accessible for the hydrogen producing bacteria. The successful utilization of these substrates therefore relies heavily on the substrate pre-treatment method (Zhang *et al*., 2007a).

Reactor configuration

Bioreactor for bio hydrogen production varies in size from small scale (100-500ml) to semi pilot scale (2-10l) and pilot scale (10-400l) which can be operated in a batch, fed batch and continuous mode(Show et al., 2011; De Gioannis *et al*., 2013). Continuous hydrogen production are more practical for large scale industrial production with several merits of monitoring and the control of process parameters, assessment of yield, substrate conversion and production rate(Ismail *et al*., 2009).

Bioreactor configurations reported in bio hydrogen production includes Continuous stirred tank reactors(CSTR) (Chen and Lin, 2003; Arooj *et al.*, 2008; Zhang *et al.*, 2007b; Kim *et al.*,

2011; Dong *et al*., 2009); upflow anaerobic sludge blanket reactors(UABR) (Chang and Lin, 2004; Gavala *et al*., 2006), anaerobic fluidized bed reactors(AFBR) (Lee *et al*., 2004; Zhang *et al*., 2008; Barros et al., 2010) anaerobic sequencing batch reactors(Vijaya Bhaskar *et al*., 2008) and membrane bioreactors (Oh *et al*., 2004; Lee *et al*., 2007; Galluci *et al*., 2013).

Among these bioreactors, CSTRs have been widely used in industrial fermentation including biohydrogen production. CSTRs are known for their effective homogenous mixing pattern resulting into a high mass transfer and substrate-microbe contact (Show *et al*., 2011). On the other hand, CSTRs application in hydrogen production are limited due to the difficulty in maintaining high levels of biomass resulting to biomass washout(Wang and Wan, 2009c).

Immobilized bioreactors are capable of retaining high biomass concentration and can be operated at shorter hydraulic retention time (HRTs) without biomass washout (Li and Fang, 2007) and high substrate conversion efficiency (Li and Fang, 2007).

Recently, membrane bioreactors are used for bio hydrogen production, they offer the benefits of low capital cost based on their small size, improvement in hydrogen yield due to equilibrium shift and low downstream processing cost as a result of integrated gas separation(Gallusci *et al*., 2013; Oh *et al*., 2004). Membrane reactors depend on the ability of the membrane to retain sludge in the mixed fermentation broth through membrane separation hence they are able to operate at a high biomass concentration (Kothari *et al*., 2012).

Temperature

Hydrogen production system can be classified into three groups based on the temperature utilized during fermentation process, they include ambient (15-30°C), mesophilic (32-39°C) and thermophilic (50-64°C). Nevertheless the comparison of these three systems is almost impossible due to variation in other process conditions such as pH, substrate and inoculum type (Kothari *et al*., 2012). Temperature is one of the most essential factors which influences the activity of the hydrogen producing bacteria particularly the hydrogenase enzyme. Reports

from literature revealed that increasing temperature within a suitable range could enhance hydrogen production although beyond certain limit further increase in temperature could decrease hydrogen production (Wang and Wan, 2008c; Lin *et al*., 2008). The optimum temperature of 35°C and 55°C have been reported for mesophilic and thermophilic bio hydrogen production respectively (Li and Fang, 2007).

Temperature can also influence the distribution of products during dark fermentation. For instance acetic acid concentration increased with increase in temperature from 20°C to 30°C in the study of Wang and Wan (2008c), further increase from 35°C to 55°C resulted to a decrease in acetic acid concentration. The results implicated the shift in the metabolic pathway and the dominance of a group of hydrogen producing bacteria at this temperature. The growth rate and the metabolic pathway of the hydrogen producing bacteria may be affected by process temperature. Beyond the optimum temperature, high temperature may cause protein denaturation thereby affecting hydrogen production (Sinha and Pandey, 2011). Some authors suggested that higher temperature may lead to improvement in hydrogen production due to the inhibition of the non spore forming hydrogen consuming bacteria (Lay *et al*., 2009).

pH

pH is considered as a vital feature of fermentative hydrogen production because of its effect on the hydrogenase system, substrate utilization and the metabolic activity of the hydrogen producing bacteria (Kothari *et al*., 2012). Reports have shown that pH affects several activities within the bacteria cells including nutrients uptake because of the sensitivity of the cell membranes (Li and Fang, 2007). Within an appropriate range, increasing pH could enhance the activity of the microbes whereas beyond the optimum value, increase in pH will affect hydrogen production. Changes in pH can alter the bacteria metabolic activity, protein synthesis, and their adaptation to unfavourable conditions (Kothari *et al*., 2012).

Controversies in terms of the optimum initial pH exist as a result of variation in other process conditions. However several authors have reported an optimum initial pH within the range of 6-7.5 (Hawkes *et al*., 2002; Khanal *et al*., 2004). Reports from literature also confirmed that initial pH can affect the duration of the lag phase during fermentation especially in batch system; an initial pH of 4 to 4.5 may contribute to an extension in the duration lag phase (Cai *et al*., 2004). Furthermore at higher initial pH, microbes produces hydrogen at a faster rate coupled with acid production which at some point affects the system buffering capacity. On the other hand, at a lower initial pH, hydrogen is produced gradually at a moderate rate for a longer duration (Sinha and Pandey, 2011).

Trace metals

Essential micronutrients are required by the microorganism for growth and metabolism during dark fermentation. These nutrients including Na, Mg, Zn and Fe are essential in hydrogen production because of their role in the bacteria enzyme cofactor, transport process and hydrogenase system (Sinha and Pandey, 2011).

Iron is a vital element for enzyme formation particularly the hydrogenase (Hawkes *et al*., 2002). A maximum hydrogen production rate of 24ml/g VSS/h was achieved with 4000mg/l $FeCl_2$ in a fermentative hydrogen production with mixed culture (Lee *et al*., 2002). Iron is very important as an electron carrier and may affect the metabolic pathway of the hydrogen producing bacteria particularly in the oxidation of pyruvate to acetyl-CoA, carbohydrate and hydrogen (Dabrock *et al*., 1992; Lee *et al*., 2002).

Apart from iron, Nickel which is a fundamental component of Ni-Fe hydrogenase also influences hydrogen production due to its involvement in the Nickel-Iron hydrogenase enzyme system. Wang and Wan reported an increase in hydrogen production rate with an increase in Ni^{2+} concentration from 0 to 0.1mg/l, however further increase in the Ni^{2+} concentration resulted in a decrease in hydrogen production(Wang and Wan, 2008b). Biogas

evolution was affected by the increase in $FeSO_4$ concentration from 13.7mg/l to 21.9mg/l resulting to a decrease in hydrogen content from 46% to 34% respectively (Lee *et al*., 2002). Magnesium functions majorly as an enzyme cofactor as well as a component of bacteria cell wall and membrane (Wang *et al*., 2007).

2.7. Challenges and prospects

Fermentative biohydrogen production currently attracts global research attention due to its sustainability and ecofriendly properties. However, biohydrogen production technology is still in a developing stage particularly its commercialization. The industrial production of this technology is faced with two major challenges of low yield and production rate as well as high cost of production (Hallenbeck and Ghosh, 2009; Lee *et al*., 2012). This may be attributed to the inability to achieve the maximum theoretical yield of 4 mole per mole of glucose (Logan, 2004; Lee *et al*., 2004). During fermentation, other by products such as butyrate, propionate, and ethanol are produced alongside hydrogen in significant amount which may contribute to the low hydrogen yield. Therefore, efforts to improve the efficiency of substrate conversion must be developed to improve hydrogen yield.

The consumption of hydrogen during fermentation by the hydrogenotrophic methanogens is considered to be another barrier facing improvement in hydrogen yield. The methanogens and homoacetogens oxidize hydrogen and reduce carbon dioxide to methane and acetate respectively (Lee *et al*., 2010). Although heat shock treatment is commonly used to combat this problem, however certain disagreement regarding the best pre-treatment method still exists hence the need for more research in this area.

The use of abundant and cheap feedstock for biohydrogen production is considered as a key factor in reducing the cost of biohydrogen production. Feedstocks such as agro waste are cheap and readily available compared to simple sugars such as glucose which are not economical for large scale industrial production (Saratale *et al*., 2008; Mtui, 2009). Recently,

research is focussed on the utilization of organic waste such as agro waste, food waste and industrial wastewater with the aim of reducing the process cost. Feedstock with low commercial value could be an attractive feedstock for biohydrogen production (Chong et al., 2009; Fernandes *et al*., 2010; Guo *et al*., 2010).

Agricultural residues are the most abundant and cheapest resources with approximately 220 billion tons annual production worldwide. Lignocellulosic biomass is rich in carbohydrate and therefore possesses great potential for biohydrogen production (Saratale *et al*., 2008).

Conversely, due to the recalcitrant nature of lignocelluloses, these feedstocks need to be treated prior to fermentation in order to alter the cell wall structure for enzyme accessibility. The resulting products (hydrolysates) containing the sugars can then be hydrolysed for hydrogen production (Ren *et al*., 2009; Ren *et al*., 2011). Substrate pre-treatment is considered as a vital step to the successful utilization of complex lignocelluosic feedstock for biohydrogen production. Therefore the quest for methods that will bring about higher substrate degradation as well as low cost should be of utmost priority.

Furthermore, the organic by products of fermentation can create environmental problems as a result of the high chemical oxygen demand(COD), for this reason the fermentation effluents need to be pre-treated before being discharged into the environment(Li and Fang, 2007). During fermentation, only about 40% of the chemical energy in the organic waste is converted to hydrogen, therefore the development of a downstream processing technology for the recovery of energy in the effluents will also help to mitigate the process cost. Some possibilities have been explored in this regard; Han and Shin (2003) evaluated the possibility of converting the residual in the hydrogen fermentation effluent to methane.

Recently, the coupling of biohydrogen production with microbial fuel cells to produce electricity was reported (Liu et al., 2005; Sekoia and Kana, 2014).

2.8. Role of Optimization in the improvement of Biohydrogen production

The optimization of fermentation conditions is an important term in the development of economically feasible bioprocesses; it is of primary importance in the development of any fermentation process owing to their impact on the economy and feasibility of the process. Optimization may be defined as the control of a process at its optimal state to reach its maximum productivity with the lowest possible cost, at the same time preserving quality (Chen *et al*., 2004). Process optimization facilitates the reduction of the cost benefit-ratio for the development industrial scale fermentation production system.

Process optimization is of central importance in industrial production processes especially in the biotechnological production processes, in which even small improvements can be vital for commercialization (Reddy *et al*., 2008; Sathish *et al*., 2008). Product formation during fermentation is influenced by many parameters which include the genetic nature of the fermenting organism, fermentation conditions, physiological parameters, nature of the substrates material and the type of bioreactor used (Screenivas *et al*., 2004; Prakasham *et al*., 2007; Lakashim *et al*., 2009; Sathish and Prakasham, 2010). Process parameters have the potential to influence the presence of bacterial groups required for the fermentation process which in turn affects the stability of the process (Krakat *et al*., 2011).

Fermentative biohydrogen production is also bound by these rules with influence by the type of substrates used (carbon source) and its concentration, the pH of the medium, reactor configuration, and other process conditions such as temperature (Wang and Wan, 2009c; Prakasham *et al*., 2009a; Fan *et al*., 2004) and nature of the inoculum used (Prakasham *et al*., 2009a). Optimization is an essential procedure to develop a more robust process for industrial application, it has been regarded as one of the most important technique to improve bio hydrogen yield (Wang and Wan, 2006; Prakasham *et al*., 2009c). Recent study on biohydrogen production reported that the pH of the fermenting medium, type of carbon

source used, concentration and age of the inoculum all plays an essential role in the overall hydrogen yield produced(Prakasham *et al*., 2009b; Chen and Chen, 2009) hence the need for optimization.

Currently, research is focused on using statistical techniques to optimize the key operational parameters that affect the process of dark fermentation. Experimental design is of immense importance in optimizing fermentative biohydrogen process because of the complexity and influence by many factors; hence an appropriate experimental design must be employed to examine the effects of these parameters (Nath and Das, 2011). Numerous method have been designed to optimize fermentative hydrogen production in which certain factors are selected and varied under a controlled manner to obtain their effects on the biohydrogen yield.

A fermentation model describes relationships between the principal variables and explains quantitatively the behaviour of the production system. The model equally provides useful suggestions for the analysis, design and operation of the fermenter (Bas and Boyacy, 2007). Modelling and optimization have been carried out with the aim of improving biohydrogen production, with the use of different modelling methods (Wang and Wan, 2009b). Owing to the shortcomings of other methods of optimization, statistical techniques such as Response Surface methodology (RSM) and Artificial Neural Networks (ANN) are increasingly being used.

2.8.1. One factor at a time design

The technique of one factor at a time is a conventional method which examines one variable singly and keeps the other parameter constant. The design entails the plotting of graphs from the result obtained to depict the effects of the single factor on the process output. Many reports on the optimization of fermentation process focused on the method of one variable at a time approach (OVAT) which is practically impossible to accomplish appropriate optimization in a finite number of experiments. Also, the OVAT

method is not very much preferred because numerous potential influential factors may be involved and their interaction could be left out (Kalil *et al*., 2000). The OVAT method is tedious and can lead to misinterpretation of results, due to its inability to show the interaction between different factors involve in the fermentation process (Haltrich *et al*., 1994).

Numerous authors have indicated the use of this design to determine the optimal conditions or parameters for biohydrogen production. For instance, Lee *et al*. (2002) studied the effect of temperature, pH and substrate concentration on fermentative biohydrogen production using one factor at a time design. T p u , s c c n c n n n d pH d n 37˚C n d 55˚C, 8-32g COD/l, 5.5-7.0 respectively and a maximum hydrogen yield of 9.47mmol H_2/g s c s n d und p c n d n s 37˚C, pH 6 0 nd 24g/COD/l of starch. In another study, Luo *et al*. (2010b) reported a maximum hydrogen yield of 53.8ml H_2 VS und p c c n d n 60˚C nd pH 6 0 cassava stillage using one factor at a time design. A 53.5% improvement in the hydrogen production yield was achieved under the thermophilic condition.

2.8.2. Response Surface methodology

Complex interactions exist among the key process parameters in any fermentation process which are often characterized using statistical techniques such as response surface methodology. Response Surface methodology (RSM) is a combination of stepwise mathematical and statistical techniques to develop, improve and optimize bioprocess. The technique require less experiment, saves time, allows flexibility to assign the levels of variables and gives closer confirmation of the output response towards the target requirements (Kenney and Krouse, 1999; Kalili *et al*., 2000). Response surface methodology consists of a group of empirical techniques committed to the assessment of associations existing between a group of experimental factors and the target

responses (Muthuvelayudham and Viruthagiri, 2007).

RSM seeks to identify and optimize significant factors with the purpose of determining the factors level that will maximize the response. The graphical representation of the model equation represents the individual and the symbiotic relationship between the process parameters on the response factor (Imandi and Rao, 2006; Wasli *et al.*, 2009). The understanding of the relationship that exists among the process variables as well as the value that can produce the optimum value of the target response are the major highlights of the RSM (Nath and Das, 2011). Examples of commonly used response surface methodology are Central composite design and Box-Behnken design (Wang and Wan, 2009b). RSM also allows minimum experimental runs whereby the levels of the operational factors are varied simultaneously with less process time

Central composite design is a five level fractional factorial design (Box and Wilson, 1951). The design generally consists of a 2^n full factorial design, 2^n axial designs and m central designs. The axial design is identical to the central design except for one factor which will take on levels either above the high level or below the low level of the 2^n full factorial design (Kuehl, 2000). The method permits the estimation of the second order polynomial and reveals the information on the relationship and interactions between the output response and the process variables (Zheng *et al.*, 2008).

Box-Behnken design is a three level fractional factorial design; including a two level factorial design with an incomplete block design. Definite numbers are put through all combinations for the factorial design in each block while other factors are kept at the central level. Box-Behnken design is more economical to use than the central composite design, because of the use of few factors and lack of too high or low levels (Wang and Wan, 2009b).

A second-order polynomial model is usually developed to describe the effects of the multiple factors on the target output based on the data obtained from the experiments. Response surface and contour map plots are usually used to display the model with the variation of only two factors levels while keeping the other factor level constant. The response surface and contour plot shows the response over the different factor levels and also the sensitivity of the response to any change in the factor with the degree of their interactions on the target response.

The model is finally subjected to analysis of variance (ANOVA) for estimation and the determination of factors with significant impact on the target output (Wang and Wan, 2009b). Optimization of biohydrogen production based on RSM has been reported by various authors as presented in Table 1.

Table 1: Some RSM Optimization research for fermentative hydrogen production

SUBSTRATE	INOCULUM	DESIGN METHOD	Factors studied	Optimum factor	Max H_2 yield	Ref
Food waste	Anaerobic sludge	RSM(CCD)	C/N ratio, inoculum conc., Na_2HPO_4 conc., endonutrient conc.	C/N ratio-33:14, inoculum conc.-2.70g VSS/L, Na_2HPO_4 conc.-6.27g/L, endonutrient conc.-7.51mL/ L	102.63mL H_2/g VS	Sreelaor *et al*., 2011
Dairy wastewater	Anaerobic sludge	RSM(BBD)	Substrate conc., pH, COD/N ratio, COD/P ratio	15.3 g COD/L, pH 5.5, COD/N ratio 100.5 , COD/P ratio 120	13.54 mmol H_2/g COD	Gadhe *et al*., 2013
Glucose	*Escherichia coli* DJT135	RSM(BBD)	pH, Tempt., substrate conc.	pH 6 5, p :35˚C ,75 M uc s	1.60mol H_2 /mol glucose	Ghosh and Hollenbeck, 2010
Municipal waste	Anaerobic sludge	RSM(BBD)	pH, Tempt., Substrate conc., HRT	pH;7 9, T p :30 29˚C, 40 45 L, 86.28 h	58.62 ml H2/g TVS	Sekoia and Gueguim Kana, 2013
Corn Stalk	Lesser Panda manure	RSM(CCD)	Tempt., SCED(g/g corn stalk), Time	T p :46 3˚C, c n s k: 0 049 , 7.5 days	205.5ml/g-TVS	Xing *et al*., 2011
Corn stover	*T. thermosaccharolyticum W16*	RSM(CCD)	Sulfuric acid conc., Reaction time	Sulfuric acid: 1.69%, Reaction time: 117mins	2.24mol H_2/ mol sugar	Cao *et al*., 2009

Glucose	*Ethanoligens harbinens W1*	RSM(CCD)	Inoculum to substrate ratio, initial pH, tempt	Inoculum:14%, initial pH:4.32, p : 34 97˚C	35.57ml/g CDW	Guo *et al*., 2011
Oil palm trunk	Hot spring sediment	RSM(CCD)	H_2SO_4conc., Reaction time, Initial pH, Total sugar conc., $NaHCO_3$ conc.	H_2SO_4:1.56w/v, Reaction time: 7.50mins. Initial pH:6.0, substrate conc.: 22.07g/L, $NaHCO_3$- 6.71g/L	1947 ml H_2/L	Khamtib *et al*., 2011
Sweet sorghum syrup	Anaerobic sludge	RSM(BBD)	Total sugar, initial pH, $FeSO_4$	Total sugar :25g/L, initial pH:4.75, FeSO4 conc.: 1.45g/L	6864 mL H_2/ L	Saraphirom and Reungsang, 2010
Molasses	Anaerobic sludge	RSM(BBD)	Substrate conc., pH, Tempt., inoculum conc.	150 L, pH: 8, T p : 35 ˚C, 10.11% inoculum ratio	71.35mL H_2/g	Whiteman and Gueguim Kana, 2013

RSM: Response surface methodology, CCD: central composite design, BBD: Box-Behnken design, H_2: Hydrogen

2.8.3. Artificial Neural Network

Bioprocess optimization based on mathematical models is described by a set of differential equations derived from mass balances. However, these models may lack robustness and accuracy as a result of the physiological complexity of microorganisms and their high versatility in terms of metabolic fluxes. The physical, chemical and biochemical requirements and characteristics of microorganism changes with time with a lot of unpredictable dynamics which makes bioprocess highly non linear (Schugel, 2001).

Recently, few numbers of studies have investigated the possibility of using non statistical technique artificial intelligence techniques to optimize fermentative hydrogen production process (Table 2). Artificial Neural Networks (ANNs) are generic description for a class of computer model which imitates the human brain functions. ANNs have the ability of modelling processes by learning from the input and output data, without the process of mathematical knowledge. They are data-driven models which can be constructed by a learning procedure which simply exposes them repeatedly to input/output data sets. The essential requirement of neural networks modelling is sufficient number of data (Imandi and Rao, 2006). ANN is similar to the behaviour of human brain neurological structures and possesses the ability to depict the interactive effects among the variables especially in multivariable non linear bioprocess.

Table 2: Some ANN Optimization studies for fermentative hydrogen production

Inoculum	Substrate	Input parameters	Output Response	Design	Reference
Buffalo dung compost	Cattle compost slurry	Medium pH, glucose to xylose ratio, inoculum age, inoculum conc.	HY	ANN-GA	Prakasham *et al.*, 2011
Anaerobic sludge	Molasses	Molasses conc., pH, temperature, inoculum conc.	CHP	ANN-GA	Whiteman and Kana, 2013
Digested sludge	Glucose	Temperature, initial pH, glucose conc.	HY	ANN-GA	Wang and Wan, 2009d
E. coli WDHL	Cheese whey	Oxidation-reduction potential(ORP), pH, dissolved CO_2	HP	BPNN	Rosales-Colunga *et al.*, 2010
Digested sludge	Glucose	Temperature, initial pH, glucose conc.	SDE, HPr, HY	NN-desirability function	Wang and Wan, 2009a
Anaerobic sludge	Sucrose wastewater	OLR, HRT, influent alkalinity	HY, HPr, HC, products conc.	ANN-GA	Mu and Yu, 2007
Activated sludge	Kitchen waste	OLR, ORP, pH, alkalinity	HP	BPNN	Shi et al., 2010
Sewage sludge	Sucrose	HRT, sucrose conc., biomass conc., pH, alkalinity, organic acid conc.	HPr	BPNN	Nikhl *et al.*, 2008

HY: Hydrogen yield, HPr: Hydrogen production rate, CHP: cumulative hydrogen production, HP: Hydrogen production, HC: Hydrogen concentration, SDE: Substrate degradation efficiency, ANN-GA: Artificial neural network-Genetic algorithm, NN: Neural network, BPNN: Back propagation neural network

Artificial neural networks (ANNs) model are designed to mimic the human learning processes by creating linkages between process input and output data. They also learn how to reproduce an output from the input parameters without any prior knowledge of the relationship between them (Rosa *et al*., 2010). ANNs have the ability to detect complex non-linear relationship between dependent and independent variables and the ability to detect all possible interaction between predicted variables hence it is considered as a non linear statistical identification technique (Subba Rao et al., 2008; Sathish and Prakasham, 2010).

ANN has a superior benefit over the response surface methodology because of its ability to model from a set of data rather than analytical description. Also, the model can be constructed solely from the process without a detailed understanding and predict outputs for new input. This is made possible because ANN can stimulate the observed properties of the biological neurological system; draw on the similarities of the biological learning with excellent generalization ability (Desai *et al*., 2006; Sathish and Prakasham, 2010). ANN represents a more accurate modelling technique due to its ability to approximate universally, i.e. it can approximate all kinds of non-linear functions including quadratic functions whereas RSM is useful only for quadratic approximations (Desai *et al*., 2008). Most ANNs consists of three layers: input, output and the hidden layer. The input layer receives information and the output layer obtains the processed results. The hidden layer can be one or a multiple layers situated between the input and the output layers (Bas et al., 2007). Artificial Neural Network (ANN) is suitable to develop bioprocess models without prior understanding of the kinetics of metabolic fluxes within the cell and the cultural environment because they are exclusively data-based (Gueguim Kana *et al*., 2012).

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CHAPTER 3

Optimization of hybrid inoculum development techniques for biohydrogen production and preliminary scale up

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Optimization of hybrid inoculum development techniques for biohydrogen production and preliminary scale up

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Inoculum pre-treatment is a crucial aspect of hydrogen fermentation processes to establish the required microbial community for hydrogen production. This paper models and opti- mizes two hybrid techniques of inoculum pre-treatment for fermentative hydrogen pro- duction: [1]pH and Autoclave (PHA); [2]pH and heat shock (PHS) using Response Surface Methodology (RSM). Coefficients of determination (R^2) of 0.93 and 0.90 were obtained for PHA and PHS respectively and the optimized pre-treatment conditions gave hydrogen yields up to 1.35 mol H_2/mol glucose and 0.75 mol H_2/mol glucose, thus a 37.75% and 15.38% improvement on model predictions for PHA and PHS respectively.

Two semi pilot scale up processes were carried out in a 10 L bioreactor seeded with the optimally treated inocula. Maximum hydrogen yields of 1.19 mol H_2/mol glucose and 0.68 mol H_2/mol glucose were obtained for PHA and PHS respectively which further verifies the practicability of this technique to enrich hydrogen producing bacteria for fermentative biohydrogen production.

1. Introduction

Current global economy and energy consumption depends mainly on fossil fuels to meet the huge energy demands [1]. These fossil fuels are non-renewable and their use contributes to greenhouse gas emissions which in turn can contribute to global warming [2]. Biohydrogen has drawn global attention recently because it is clean, renewable and inexhaustible with low carbon emission [3]. It is an ideal alternative to fossil fuels because of its ability to eradicate the problem of greenhouse gas emission. Hydrogen has been efficiency and non-polluting characteristics [4]. In addition, hydrogen gas has been proposed as the ultimate transport fuel for vehicles and vessels because of the use of highly efficient fuel cells to convert chemical energy to electricity [5].

Fermentative hydrogen production can be performed by pure and mixed microbial cultures [6]. However, the use of pure cultures in dark fermentation is potentially more expensive than employing mixed cultures as it requires sterile conditions and strict process control [7]. At the same time the use of mixed cultures is prone to deliver lower hydrogen yields due to the presence of H_2 consuming

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[1] PHA e pH and Autoclave.

Inoculum composition can greatly affect the efficiency of hydrogen production. Inoculum pre-treatment can inhibit the non-hydrogen producing and hydrogen consuming popula- tion within the mixed microbial community [9]. In addition, it selects a suitable microbial hydrogen producing population [10] for the start-up and safeguards process stability for a continuous hydrogen production [11]. Various inoculum pre- treatment methods including heat shock, acid or base treat- ment, aeration, freezing and thawing and use of methanogen inhibitors such as chloroform have been reported [9,10,12e17]. Some studies have also indicated that process parameters such as low pH [18] and hydraulic retention time (HRT) [19] can inhibit methanogens.

Heat treatment selects for bacteria that are spore formers such as Clostridium spp., Bacillus spp. thereby resulting in a microbial community which is relatively homogenous compared with non-heat treated inocula [20]. However, the use of heat treatment is limited in that hydrogen producing bacteria which are non-spore formers such as Enter- obacteriaceae and genera within this family such as Citrobacter spp. [21,22] will be affected. Also heat treatment does not select only the hydrogen producing bacteria because homo- acetogens and lactate producers such as Sporolactobacillus spp. present in the mixed culture may not be affected as they possess the ability to form endospores [20].

Response surface methodology consists of a group of empirical techniques devoted to the evaluation of relation- ships existing between a cluster of controlled experimental factors and the measured responses [23]. RSM seeks to identify and optimize significant factors with the purpose of determining what levels of the factors maximize the response, unlike the one factor at a time method which can lead to misinterpretation of results because of its failure to show the interaction between different factors involved in the fermentation process [24]. Response surface methodol- ogy has been successfully used for the optimization of several fermentation processes including hydrogen produc- tion [25,26].

This paper modeled and optimized two hybrid pre-treatment techniques for biohydrogen inoculum develop- ment namely pHeAutoclave (PHA) and pHeHeat shock (PHS). The interactive effects of pH and autoclaving time as well as pH, heat shock time and heat shock

2. Materials and

2.1. Seed sludge

Anaerobic sludge used as the inoculum in this study was ob- tained from the Darvill Waste Water treatment plant in Pie- termaritzburg, South Africa. The sludge was immediately transferred to the laboratory and stored at 4 C. Prior to use the sludge was sieved through 2 mm grain size matters. The sludge pH and volatile suspended solids (VSS) were 7.35 and 10.23 g/l respectively.

2.2. Pre-treatment

The experiments were designed to evaluate the influence of the hybrid inoculum pre-treatment techniques on hydrogen production efficiency. Two pre-treatment

2.3. Experimental design and inoculum development

2.3.1. pH and autoclave (PHA)

The RSM Central Composite Design (CCD) was adopted to develop the PHA hybrid technique. The independent variables consisted of pH and autoclave duration in the range of 2e12 and 1e15 (min) respectively, and hydrogen yield was the response index. A total of thirteen runs (Table 1) were carried out according to the CCD and the centre points were repli- cated four times to estimate the experimental errors and for reproducibility. The CCD

$$Y ¼ b_0 þ \sum b_i X_i þ \sum b_{ii} \$ X^2_i þ \sum b_{ij} \$ X_i \$ X_j \quad (1)$$

where Y is the hydrogen yield, X_i are the input variables, b_o is a constant; b_i the linear coefficient; b_{ii} the squared coefficients and b_{ij} the cross product coefficients.

The PHA pre-treatment was carried out by adjusting the pH

2.3.2. pH and heat shock (PHS)

The RSM Box-Behnken design was adopted for the PHS hybrid technique. pH, heat shock temperature and heat shock duration were the independent variables selected in the ranges of 2e12, 80 Ce100 C and 15e120 (min) respectively. Seventeen fermentation batches with varied the considered were generate (Table 4) and

Table 1 e Hydrogen yield from pH and autoclave hybrid inoculum treatments.

Run	pH		Autoclave time (min)		H_2 yield
	A	Code	B	Code	(mol H_2 mol^{-1} glucose)
1	11.00	1	15.00	1	0.96
2	7.00	0	9.00	0	0.81
3	7.00	0	9.00	0	0.77
4	7.00	0	9.00	0	0.88
5	11.00	1	3.00	1	1.01
6	1.34	a	9.00	0	0.30
7	7.00	0	17.49	a	1.1

evaluated for hydrogen response. A quadratic function was

fitted to the experimental data (as stated above in Eq. (1)).

The PHS pre-treatment was achieved by adjusting the pH of the sludge with 1 M HCl and 1 M NaOH, maintained for 24 h followed by heat treatment at varying temperature duration according to the Box-Behnken design.

2.4. Batch fermentation

The anaerobic sludge was subjected to the pre-treatment

procedures with varied conditions as described in the RSM designs, and assessed for hydrogen production efficiency. Fermentation experiments were conducted in triplicate in a modified 250 ml Erlenmeyer flask and each flask contained

20 ml of the treated sludge and 180 ml of a defined medium to make a total working volume of 200 ml. The defined medium contained 10 g/L glucose as the carbon source and the following inorganic salts (g/L): NH_4Cl 0.5; KH_2PO_4 0.5; K_2HPO_4

0.5; $NaHCO_3$ 4.0; $FeCl\$4H_2O$ 0.15; $MgCl\$6H_2O$ 0.085;

$ZnSO_4\$7H_2O$ 0.01; $MnCl_2\$4H_2O$ 0.03; H_3BO_3 0.03; $CaCl_2\$6H_2O$

0.01; $Na_2MoO_4\$2H_2O$ 0.03. The initial pH value of the medium

2.5. Scale-up experiment

Pilot processes were carried out in a 10 L bioreactor (Labfors-INFORS HT, Switzerland). The reactor was heat sterilized, fed with medium as previously described and inoculated at 10% (v/v) with a 48 h old optimally pretreated inoculum and a total working volume of 7 L. The bioreactor control set points of pH, temperature, agitation and process time were set at 5.5, 35 C,

150 rpm and 72 h respectively. Throughout the fermentation process, the evolving gas was streamed through an array of sensors (Bluesens, Germany) to

2.6. Analytical methods

The volume of evolved gas was measured daily using water

displacement method and the hydrogen concentration was determined every 6 h with a hydrogen sensor (BCH- H_2 Blue- sens, Germany). The sensor has a measuring range of 0e100% H_2 and uses the thermal conductivity measuring principle. The cumulative volume of hydrogen was computed recur- sively using the fraction hydrogen in the

$$V_{H;i} ¼ V_{H;i\ 1} þ C_{H;i}\ V_{G;i}\quad V_{G;i\ 1}\ þ V_H\ C_{H;i}\quad C_{H;i\ 1} \tag{2}$$

$V_{H,i}$ 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

intervals, $C_{H,i}$ and $C_{H,i\ 1}$ the fraction of hydrogen gas in the headspace of the bottle in the current and previous time in- tervals, and VH the total volume of headspace in the bottle.

The molar hydrogen yield was calculated by dividing the cumulative volume of hydrogen (at standard temperature

$$HY ¼ N_{H;F}= N_{g;i}\quad N_{g;f} \tag{3}$$

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.

Volatile suspended solid (VSS) was determined according to standard methods [28]. Samples of the medium were taken after fermentation in order to measure the residual glucose concentration using a glucose analyzer (Model 2700 select, YSI USA). pH was measured by a pH meter (Crison, South Africa). Volatile fatty acids concentrations (VFA) were determined using a gas chromatograph (Model YL6100, Younglin South Korea) equipped with a flame ionization detector (FID) and a capillary column (30 m 0.25 mm 0.25 mm). The fermenter sample was first centrifuged at 17,664 g for 10 min and acidified with 1 M HCl to pH 2 after which the supernatant was filtered through 0.45 mm membrane. Nitrogen gas was used as a carrier gas at a flow rate of 30 ml/min and a split ratio of 1:50. The operational temperature of the injection port and detector were 220 C and 260 C respectively and the initial tempera- ture of the oven was set at 125 C for 5 min then

2.7. Model validation

Validation experiments for the obtained PHA and PHS models were carried out by treating the inoculum according to the predicted optimal conditions (Table 6) followed by their assessment for hydrogen production. All tests were per- formed in triplicate

3. Results and discussion

3.1. Modeling and optimization of pH and hybrid pre-

Experimental data obtained from CCD were used to develop a

second-order polynomial equation (Eq. (4)) whose

$$Y ¼ 0:82\ þ 0:18A\ þ 0:073B\quad 0:045AB\quad 0:14A\ þ 0:068B\ ^2 \tag{4}$$

where Y is the hydrogen molar yield, A and B are the linear coefficients, AB represents the interactive coefficients of pa- rameters on hydrogen production, A^2 and B^2 are the quadratic coefficients.

The suitability of the model was assessed by Analysis of Variance (ANOVA) (Table 2). This analysis compares the vari- ation due to residual with the variation of data about the

Table 2 e Analysis of variance for PHA model.						
Source	Statistics	df	Mean	F-value	P-value	R^2
Model	50.70	5	10.14	19.58	0.0005	0.9333

df: Degrees of freedom, F-value: FishereSnedecor distribution value, P-value: probability value, R^2: coefficient of determination.

used to predict the response can be established using Fish-

ereSnedecor distribution (F-test) and probability value (P-value). The high model F-value (19.58) and low P-value (0.0005) implies that the model is significant. The model equation showed a coefficient of determination (R^2) of 0.933 indicating that the model can explain 93% of the variation in the response observed which confirms the significance of the model. The coefficient of estimates are shown in Table 3, where A and B are the linear coefficients of pH and autoclave time, AB is the interactive coefficient of pH and autoclave time, A^2 and B^2 are the square terms of pH and autoclave time respectively.

The three dimensional response surface curve and corre- sponding contour map plots (Fig. 1A and B) showed a clear peak which indicates that the optimum pre-treatment con- ditions of PHA can be obtained within the design boundaries.

The maximum hydrogen yield was achieved at pH 8.8 and autoclave treatment of 15 min. However, an increase of autoclave time, or pH above these threshold values did not increase hydrogen yield. This could be attributed to the PHA treatment enriching for endospore forming hydrogen pro- ducing bacteria while at the same time inhibiting hydrogen utilizing methanogens present in the mixed culture. Hydrogen producing endospore forming bacteria have the ability to survive unfavorable conditions [29] as endospores are very resistant to heat,

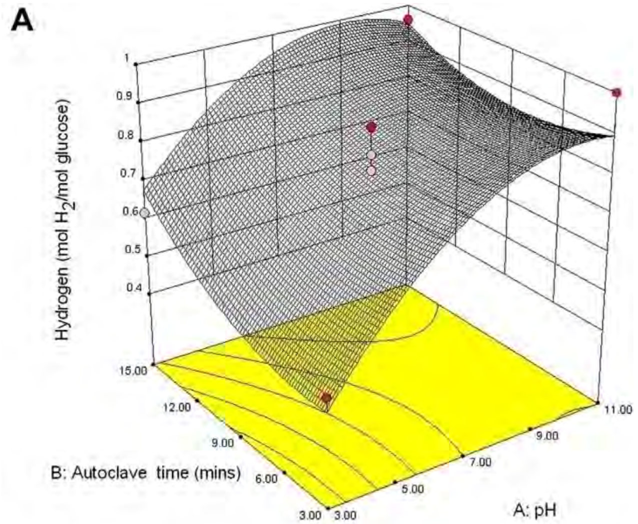
3.2. Modeling and optimization of pH and heat hybrid pre-

The data obtained from the Box-Behnken design was fitted to

a second-order polynomial equation (Eq. (5)) which defines

Table 3 e Coefficients of estimates of the mixture model and their confidence intervals.					
Component	Coefficient	df	Standard	95%	95%
Intercept	8.21	1	0.32	7.45	8.97
A	1.82	1	0.25	1.22	2.42
B	0.73	1	0.25	0.12	1.33
A^2	1.42	1	0.27	2.06	0.77
AB	0.43	1	0.36	1.28	0.42

df: Degrees of freedom, 95% CI low: 95% confidence intervals (low limit), 95% CI high: 95% confidence intervals (high limit).



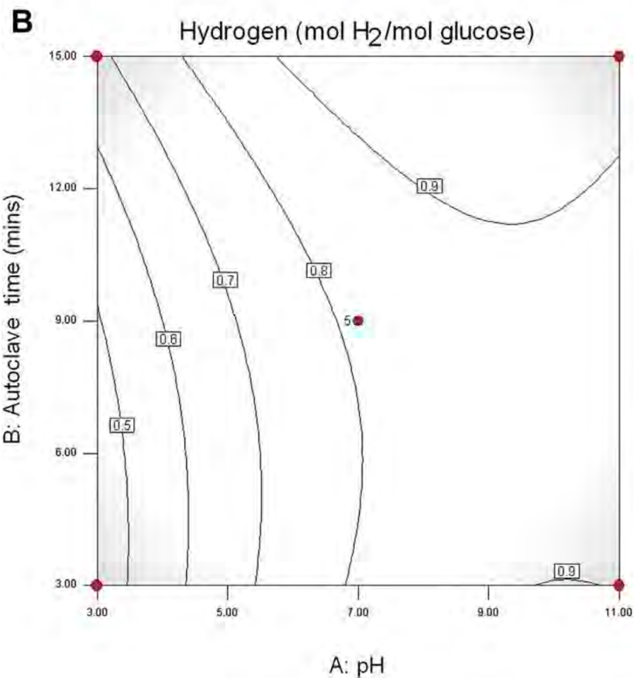


Fig. 1 e A and B: Response surface and contour map plots of PHA model showing the interactions of pH and autoclave duration on hydrogen molar yield.

Y ¼ 0:65 þ 0:037A 8:750E 0:003B 0:011C 0:027AB 0:063AC 0:030BC 0:092A^2 0:055B^2 0:080C (5)

where Y represents hydrogen molar yield, A, B and C represent pH, heat shock time and heat shock temperature, respectively.

The result of the Analysis of variance (ANOVA) of the quadratic model is summarized in Table 5. The regression equation obtained from the ANOVA indicated a coefficient of determination (R^2) of 0.90 indicating that 90% variations in the observed data can be explained by the model.

Table 4 e Hydrogen yield from pH and heat shock hybrid inoculum treatments.

Run	pH		Heat time (min)		Heat temp (C)		H_2 yield (mol H_2 mol [1] glucose)
	A	Code	B	Code	C	Code	
1	7.00	0	67.50	0	90.00	0	0.64
2	7.00	0	120.00	1	100.00	1	0.48
3	2.00	1	15.00	1	90.00	0	0.48
4	12.00	1	67.50	0	80.00	1	0.57
5	7.00	0	67.50	0	90.00	0	0.64
6	12.00	1	120.00	1	90.00	0	0.47
7	2.00	1	67.50	0	80.00	1	0.37
8	7.00	0	15.00	1	80.00	1	0.49
9	7.00	0	67.50	0	90.00	0	0.67

which confirmed that the model can be used to navigate the design space. The model's coefficient of estimates are shown in Table 6, where A, B and C, are the linear coefficients for pH, heat shock time and heat shock temperature respectively. The most significant model's terms were A, AC, A^2, B^2, and C^2.

The three dimensional response surface and contour map plots showing parameter interactions pair wise are presented in Fig. 2AeF. The response surface curves were convex in shape confirming that the optimum conditions were well defined and the combined effects may vary slightly from the single variable conditions. The shapes of response surface and contour plots indicate the nature and extent of the interaction between the input variables and the desired response [35].

The interaction between pH and heat shock time (Fig. 2A and B) showed that there was an enhancement in the hydrogen yield up to 68 min of heat treatment and further increase led to its decline. This was consistent with reports that short period of heat treatment (15 min) produced rela- tively low hydrogen yields [36,37]. In the work of Lee et al. [38], a maximum hydrogen yield of 1.55 mol H_2/mol glucose was recorded with the heat pre-treatment of sewage sludge for 1 h. Maximum hydrogen yield was

Table 5 e Analysis of variance for PHS model.

Source	Statistics	df	Mean	F-value	P-value	R^2
Model	0.12	9	0.013	7.29	0.0079	0.9036

df: Degrees of freedom, F-value: FishereSnedecor distribution value, P-value: probability value, R^2: coefficient of determination.

Table 6 e Coefficients of estimates of the mixture model and their confidence intervals.

Component	Coefficient estimat	df	Standard error	95% CI low	95% CI high
Intercept	0.65	1	0.019	0.60	0.70
A	0.037	1	0.015	0.0018	0.073
B	0.00875	1	0.015	0.044	0.027
AB	0.027	1	0.021	0.078	0.023
C	0.011	1	0.015	0.047	0.024
BC	0.030	1	0.021	0.080	0.020
B^2	0.055	1	0.021	0.10	0.00586
AC	0.063	1	0.021	0.11	0.012

df: Degrees of freedom, 95% CI low: 95% confidence intervals (low limit), 95% CI high: 95% confidence intervals (high limit).

Oh et al. [22] also reported that increased duration of heating may be effective in eliminating hydrogen consumers as spore forming homoacetogenic bacteria such as Clostridium aceticum [40] may still be present in the mixed culture after short heat treatment; resulting in hydrogen consumption for acetic acid production.

The interaction between pH and heat shock temperature (Fig. 2C and D) confirmed that heat shock temperature has a major impact in the pre-treatment of inocula for hydrogen production. As seen in the response surface curve, hydrogen molar yields increased with increase in pH as well as heat temperature. This shows that the hydrogen yield is influenced by the pH and temperature during heat treatment which may be a result of the elimination of the non-spore forming hydrogen consuming bacteria and initiation of spore germi- nation in Clostridium spp through the alteration of their germination receptors [41,42].

Further increase in the two factors led to significant decrease in hydrogen yield, one possible reason for this may be that the non-spore forming hydrogen producing bacteria such as Enterobacter spp were destroyed as a result of the heat treatment. This observation was consistent with several reports; cumulative hydrogen yield decreased faster when the pre-treatment temperature exceeded 80 [43] while Baghchehsaraee et al. [44] reported a decrease in hydrogen production yield as the temperature increased above 89 C. However, heat shock time and heat shock temperature (Fig. 2E and F) did not show

3.3. Optimization of pre-treatment

Optimization by a 'one at a time' approach is not only laborious and time consuming but also has the limitations of ignoring the importance of interactions of the various pro- cess parameters that can affect hydrogen production. The response surface methodology used in this study shows the importance of combining pro-

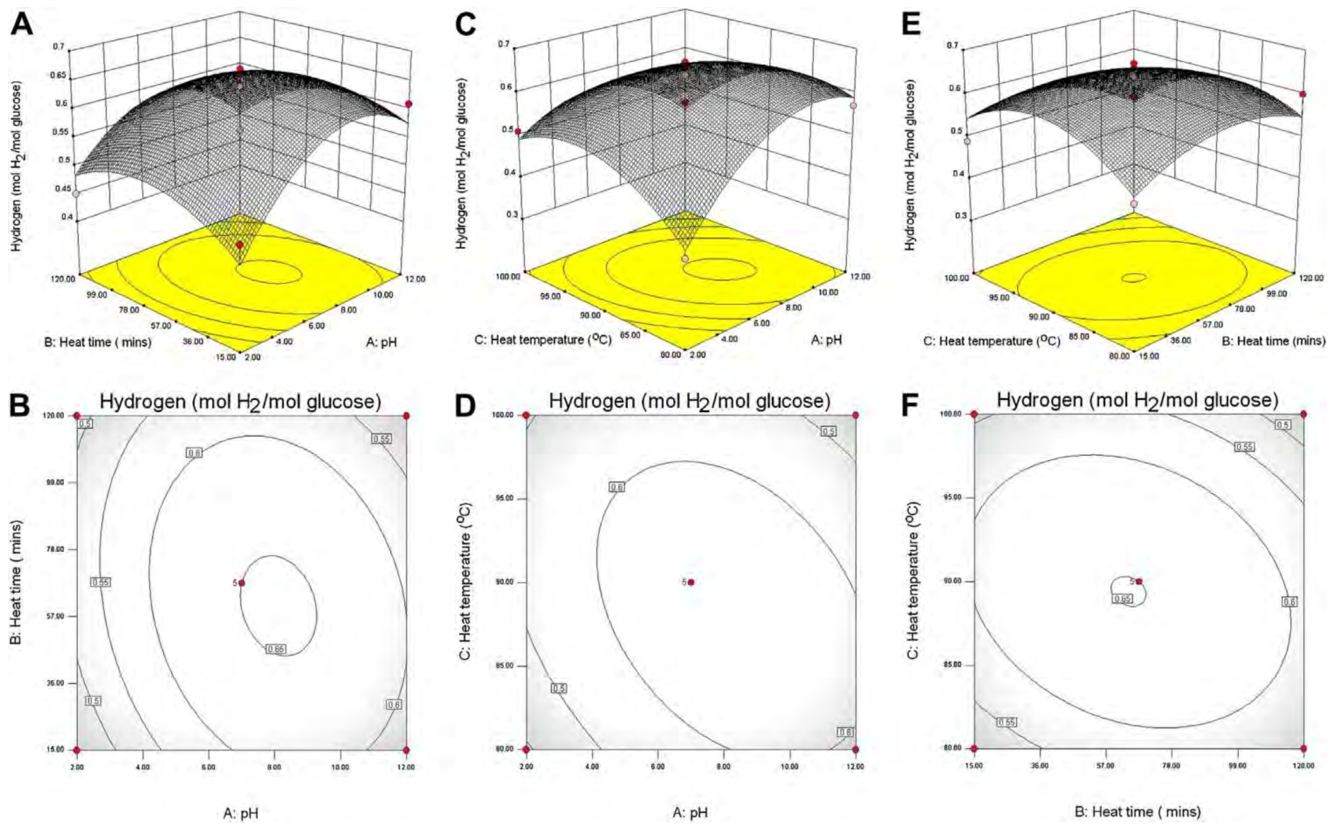


Fig. 2 e A and B: Response surface and contour map plots of PHS for maximum hydrogen yield showing the interaction between pH and heat time. C and D: Response surface and contour map plots of PHS for maximum hydrogen yield showing the interaction between pH and heat temperature. E and F: Response surface and contour map plots of PHS for maximum hydrogen yield showing the interaction between heat time and heat temperature.

enhance hydrogen production through the deactivation of hydrogen consuming methanogens present in the inoculum.

The obtained quadratic equations for both models were solved by the method of Myers and Montgomery [46] to obtain the optimum operational set points for inoculum develop- ment which are shown in Table 7. Optimization of PHA treatment technique suggested a combination of pH value and autoclave time of 8.93 and 15 min respectively, which gave a hydrogen yield of 1.35 mol H_2/mol glucose. For PHS, an optimal combination of pH value and heat shock treatment of

Table 7 e Optimized hybrid inoculum treatment conditions.					
	pH	Autoclave duration (min)	Heat temperature (C)	Heat time (min)	Hydrogen yield (mol H_2/mol
PHA	8.93	15	e	e	0.98
PHS	8.36	e	89	68	0.65

3.4. Preliminary process scale up with optimized inocula

Fermentation scale up is aimed at the production of larger product quantities ideally with a simultaneous increase or at least consistency of specific yields and product quality [47]. The scale up experiment was performed using the optimized pretreated conditions and the biogas evolution trends for the two hybrid pre-treatment techniques are shown in Fig. 3A and B. As shown in Fig. 3A, hydrogen production began after a lag period of 18 h, with hydrogen and carbon dioxide showing the same pattern of evolution for the two processes. The lengthy lag phase of 18 h may be accounted for by the harsh conditions of heat treatment, the need for spore germination as well as time required to adapt to new conditions and synthesize en- zymes for replication and metabolism.

The performance of the pilot scale up system was evaluated based on the cumulative hydrogen production and hydrogen yield. Gas produced composed mainly of hydrogen and carbon dioxide, with hydrogen yield of 1.19 mol H_2 mol ^{1} glucose and 0.68 mol H_2 mol ^{1} glucose obtained for PHA and PHS respec-

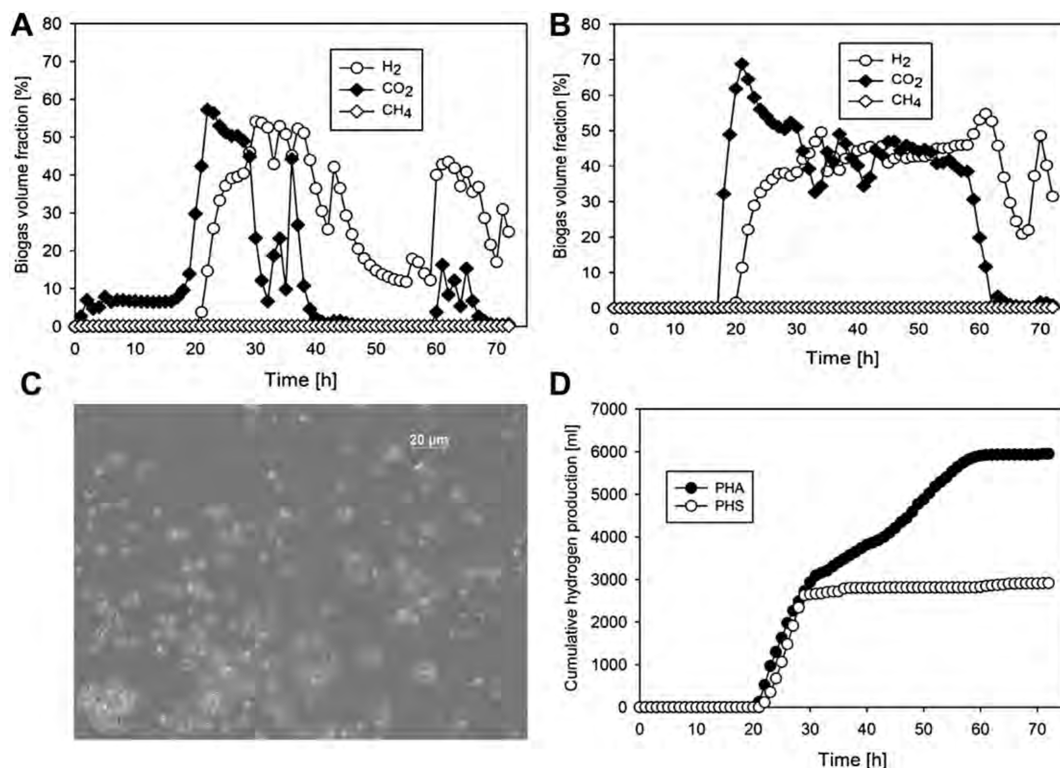
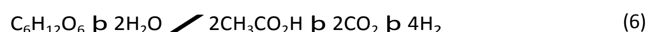


Fig. 3 e A: Biogas composition with PHA pre-treatment scale up process. B: Biogas composition with PHS pre-treatment scale up process. C: Presence of endospore forming clostridia in a reactor sample demonstrated by phase contrast microscopy. D: Cumulative hydrogen production for PHA and PHS scale up processes.

hydrogen production by Ren et al. [48], the biogas comprised mainly of carbon dioxide and about 40e50% hydrogen fraction.

The cumulative volume of hydrogen produced (Fig. 3D) with the PHA treated inoculum (5.949 L) was higher compared to process with PHS treated inoculum (2.912 L); this observation could be a result of the presence of hydrogen consuming microbes such as endospore forming homoacetogens which were not completely inhibited in the PHS inoculum [16]. The presence of non-hydrogen producing carbohydrate con- sumers will have a negative impact on the overall perfor- mance of the hydrogen production system as they compete with the hydrogen producing microorganisms thereby reducing hydrogen yields [49].

The analysis of volatile fatty acid concentration from both systems showed that butyric acid and acetic acid were the major VFAs produced during the fermentation process. The dominant metabolite was butyric acid accounting for 76.8% for PHA and 84.8% for PHS. Acetic acid was



$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CO_2H + 2CO_2 + 4H_2 \quad (6)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2CO_2H + 2CO_2 + 2H_2 \quad (7)$$

Thus the theoretical hydrogen yield is 4 mol of hydrogen per mole of glucose for the acetic acid type fermentation (Eq. (6)) and 2 mol of hydrogen per mole of glucose for the butyric acid type fermentation (Eq. (7)).

The results obtained from both process (PHA and PHS) scale up suggested the establishment of butyric acid type fermenta- tion based on the dominance of butyric acid. On the contrary, Wang et al. [45] reported a mixed acid type fermentation by heat shock pretreated inoculum (80 C and 90 C) with the production of acetic acid, butyric acid and ethanol. Acetate accounted for about 71e74% of the total VFAs produced. This may be due to the influence of pH in the hybrid pre-treatment techniques, as the fermentation pathway will be affected by the pre-treatment of the inoculum [42]. The composition of metabolites produced during hydrogen fermentation is an important indicator of the performance of hydrogen producing microorganism present in the inoculum. Controlling the hydrogen

4. Conclusion

Response surface methodology was used to study the inter- active effects of pH and autoclave duration as well as pH, heat temperature and heat time for the deactivation of hydrogen consuming bacteria and enhancement of hydrogen producers in a mixed culture inoculum.

The models suggested and confirmed upon validation that hybrid inoculum treatments at a pH value of 8.93 followed by autoclaving (121 C) for 15 min, or at pH of 8.36 followed by heating at 89 C for 68 min led to a maximum hydrogen yields of 1.35 mol H_2/mol glucose and 0.78 mol H_2/mol glucose. Preliminary scale up for both models was carried out with investigations on the biogas composition, volatile fatty acids concentration and microscopy demonstration of the presence of presumptive Clostridium spp. The biogas composition revealed up to 53% hydrogen content and butyric acid type fermentation as butyrate was the main VFA produced. Methane production was effectively inhibited in both systems and the presence of Clostridium spp. as potential hydrogen producing microorganisms was confirmed. Hydrogen molar yields of 1.19 mol H_2/mol glucose to enrich hydrogen producing microorganism in mixed inocula.

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CHAPTER 4

Optimization of biohydrogen inoculum development via a hybrid pH and microwave treatment technique- Semi pilot scale production assessment

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Optimization of biohydrogen inoculum development via a hybrid pH and microwave treatment technique e Semi pilot scale production

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abstract

The interactive effect of a hybrid pH and microwave pre-treatment on a mixed inoculums for biohydrogen production was investigated. Response surface methodology (RSM) was employed to obtain the optimum pre-treatment conditions of pH, microwave duration and microwave intensity for maximum hydrogen yield. The obtained model had a coefficient of correlation (R^2) of 0.87. The optimum inoculum pre-treatment conditions predicted were pH 11 and 2 min microwave treatment at 860 W and the validation experiments demonstrated a 32.41% increase on hydrogen yield.

Two semi pilot scale-up processes were carried out in a 10 L bioreactor initiated in batch mode with 7 L working volume using the optimally pre-treated inoculum. In the absence of pH control, 46% of glucose was utilized corresponding to a molar hydrogen yield of 1.78 mol H_2/ mol glucose and a maximum hydrogen fraction of 49.3% whereas under controlled pH envi- ronment, a twofold increase in glucose utilization was obtained which corresponds to a molar hydrogen yield of 2.07 mol H_2/mol glucose and a maximum hydrogen concentration of 56.4%.

The controlled fermentation significantly improved biohydrogen production in the scale-up process and methane production was completely suppressed suggesting the effectiveness of the combined pre-treatment to enrich hydrogen producing bacteria. Viable counts and microscopical analysis indicated the presence of hydrogen producing endo- spore forming presumptive Clostridum species.

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1. Introduction

Increasing energy prices together with the current climate change debate have placed a high demand on cleaner and sustainable forms of energy. Hydrogen is believed to be an environmentally friendly source of clean and renewable energy [1] and currently research is focused on its sustainable biotechnological production. It has been estimated that the contribution of hydrogen to total energy basket will be 8e10%

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by 2025 [2]. Among the different biohydrogen production technologies, fermentative hydrogen production is very attractive with a wide range of potential waste materials to be utilized as substrates [3e6]. Dark fermentation remains an environmental friendly and feasible method of producing hydrogen by generating low quantities of green house gases; simultaneously utilizing solid waste and wastewater through the activity of the hydrogen producing heterotrophic micro- organisms [7]. Although biohydrogen production has a great potential for sustainable energy production, this technology is still faced with two major challenges, low yield and purifica- tion, these obstacles needs to be addressed to speed up the realization of a viable hydrogen economy [4,8]. Various tech- niques such as chemical adsorption, cryogenic distillation and more recently membrane technology have been developed for integrated biohydrogen production and purification [9].

Biohydrogen production using mixed cultures has several potential benefits which include simplicity of operation as there is a low requirement for sterile conditions and better adaptation capability regarding substrates and physico- chemical conditions as a result of the microbial diversity and high capacity for continuous processing [10e13]. At the same time, the species composition and diversity of the hydrogen producing microbial population will in turn be affected by the pre-treatment method applied. Therefore an appropriate inoculum pre-treatment can contribute a great deal to increased hydrogen yields [14].

Not surprisingly, numerous studies reported the effects of different inoculum pre-treatment methods for fermentative hydrogen production including exposure to alkaline, aeration, heat, acid, microwave, sonication, methanogenesis inhibitors (such as chloroform), and combinations thereof [14e20], with the ultimate goal of improving hydrogen yields.

Microwave pre-treatment of anaerobic sludge for bio- hydrogen generation has been scantily reported [21]. Howev- er, this method has been applied for many processes such as contaminated soil remediation, waste processing and sanita- tion, food sterilization and organic or inorganic synthesis [22e25]. Electromagnetic radiation generated via the micro- wave treatment falls typically into the frequency range of

0.3e300 GHz [23].

Stable and continuous hydrogen production by an anaerobic mixed community including active hydrogen producing bacteria together with the suppression of hydrogen consuming microorganisms are essential tools for long term and industrial scale fermentative hydrogen production [14]. Despite numerous studies on inoculum pre-treatment tech- niques for anaerobic sludge at laboratory scale (typically up to

2. Materials and

2.1. Inoculum

Anaerobic sludge used as the inoculum in this study was ob- tained from the Darvill WWT in Pietermaritzburg, South Af- rica. The sludge was immediately transferred to the laboratory and stored at 4 C. Prior to use, the sludge was filtered through a 20 mesh sieve to remove large solid

2.2. Experimental design

The RSM Box-Behnken design was used for model develop- ment and to optimize the hybrid pre-treatment technique for biohydrogen production. It is a three level design based on the combination of two-level factorial and incomplete block de- signs with excellent predictability within the design space and requires a lesser number of experiments than the full factorial design [26].

The three independent variables pH (A), microwave treat- ment duration (B) and microwave intensity (C) were consid- ered as input parameters. These input parameters were varied in the range of 2e12, 2e5(min) and 500e1000(W) for pH, mi- crowave duration and microwave intensity respectively (Table 1).

A total number of seventeen fermentation experiments were carried out according to the design and the center points were replicated five times to estimate the

$$Y = b_o + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum b_{ij} X_i \quad (1)$$

Where Y is the predicted response (hydrogen yield); b_o a constant: b_i the linear coefficient: b_{ii} the squared

Table 1 e Box-Behnken design with the observed

Run	pH		Microwave duration (min)		Microwave intensity (Watt)		H_2 yield
	A	Code	B	Code	C	Code	(mol H_2 mol^{-1}
1	12.00	1	3.5	0	500	1	0.69
2	2.00	1	3.5	0	500	1	0.52
3	7.00	0	3.5	0	750	0	0.60
4	7.00	0	3.5	0	750	0	0.64
5	12.00	1	5	1	750	0	0.78
6	12.00	1	2	1	750	0	1.28
7	7.00	0	5	1	500	1	0.67
8	2.00	1	5	1	750	0	0.50
9	7.00	0	3.5	0	750	0	0.60

and b_{ij} the cross product coefficients; and X_i and X_j are the input variables. The fitness of the model was assessed by the Analysis of Variance (ANOVA) using Design-Expert 8 software (Stat-Ease, Inc. USA).

2.3. Hybrid inoculum pre-treatment procedure

A hybrid method with pH and microwave was used to pretreat

the inoculum in order to select for hydrogen producing an- aerobes. The pH treatment was carried out by adjusting the pH of the sludge with 1 M HCl and 1 M NaOH solution using a calibrated pH electrode (Crison, South Africa) maintained for

24 h. This was followed by the microwave treatment at varying duration and intensity levels according to the RSM design. The microwave treatment was conducted using a domestic mi- crowave oven (Model DMO353 Defy, South Africa). The micro- wave system has a maximum power

2.4. Batch fermentation

Fermentation experiments were carried out in a modified

250 ml Erlenmeyer flasks with a working volume of 200 ml. Each flask was inoculated with 10% (v/v) pre-treated inoculum and a previously reported defined medium [19] under aseptic condition. The initial pH was adjusted to 6.0 by adding 1 M HCl or 1 M NaOH solution, the flasks were flushed for 3 min with nitrogen to provide anaerobic conditions and capped tightly with rubber stoppers. The fermentation process was carried out at 35 C in a shaker water bath at 150 rpm for 72 h. Each run was carried out in triplicate and the

2.5. Analytical methods

Hydrogen concentration and the gas volume were determined

with a previously reported hydrogen sensor (BCH- H_2 Blue- sens, Germany) [19,27] in combination with the water displacement method [19]. The cumulative volume of

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

$V_{H,i}$ and $V_{H,i-1}$ are cumulative hydrogen gas 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 in- tervals, $C_{H,i}$ and $C_{H,i-1}$ the fraction of hydrogen gas in the headspace of the bottle in the current and previous time in- tervals, and VH the total volume of headspace in the reactor.

The molar hydrogen yield was calculated by dividing the cumulative volume of hydrogen (at standard temperature

$$HY = N_{H,F} / \left(N_{g,i} - N_{g,f}\right) \quad (3)$$

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

Volatile suspended solids (VSS) were determined according to APHA standard methods [28] while the total chemical ox- ygen demand (tCOD) and the soluble chemical oxygen de- mand (sCOD) were analyzed using a COD test kit (Merck, Germany) as reported previously [29]. For sCOD, the sample was first filtered through 0.45 mm filter. pH was measured by using a pH meter (Crison, South Africa) while volatile fatty acids (VFAs) and ethanol were determined by GC-FID as re- ported previously [19]. The residual glucose concentration was determined using a glucose analyzer (Model 2700

2.6. Pilot scale-up experiment

Two semi pilot scale fermentation processes with different pH

control schemes were carried out. Prior to the scale-up experiment, a batch fermentation was carried out using a modified 1 L Erlenmeyer flask with a total volume of 700 ml to build up biomass concentration. The flask was inoculated with 70 ml optimized pre-treated sludge and fed with 630 ml of the defined medium. pH, temperature and agitation were set at 6.0, 35 C and 150 rpm respectively. The system was flushed with nitrogen gas for 5 min and the fermentation process was conducted for 24 h.

The pilot scale fermentation process was carried out in an autoclaved 10 L bioreactor (Labfors-INFORS HT, Switzerland) containing 10 g/l glucose (i.e. 55.55 mM) to a working volume of 7 L. It was inoculated at 10% (v/v) with optimally pre-treated sludge with a combination of microwave and pH. For the pilot process without pH control, the initial pH of the culture me- dium was adjusted to 6.0 by adding 1 M HCl or 1 M NaOH so- lution. The control set points of temperature, and agitation speed and process time were set at 35 C, 150 rpm and

96 h followed by nitrogen flushing for 10 min to ensure anoxic

2.7. Pilot scale process monitoring

The biogas evolving from the process was passed through an

array of three sensors to determine the fraction of hydrogen, methane and carbon dioxide. The gas sensors used were (BCPeH_2), (BCPeCH_4) and (BCPeCO_2) with measuring ranges of (0e100 vol.%), (0e100 vol.%) and (0e50 vol.%) respectively (Bluesens, Germany). The evolving biogas volume was moni- tored using a milligas counter (MGC, Bluesens, Germany). These sensors were interfaced to the Flab-Biogas software described in our previous study [30] and the sampling interval was set to 1 min.

The concentration of volatile fatty acids (acetate,

2.8. Microbial counts and microscopy

Samples were taken periodically from the bioreactor during

the pilot scale process and analyzed by phase contrast

using sterile ringer solution. Differential reinforced clostridia medium (DRCM) [31] was used for the enumeration of pre- sumptive Clostridium spp. using 1 ml samples (in triplicate) from the decimal dilutions and the pour plate technique while anaerobic plate counts were established using plate count agar (Merck, Germany). Plates were incubated for 72 h at 37 C (DRCM) and at 35 C (PCA) in an anaerobic jar (OXOID). Phenotypic confirmation based on the Gram stain, endospore detection, catalase, oxidase and motility test were done for ten randomly selected colonies from at least

3. Results and discussion

3.1. Model analyses and

Hydrogen responses under the varied inoculum pre- treatment

conditions are presented in Table 1. The experimental data were fitted to a second order polynomial equation (Equation (4)) which defines the predicted response(Y) in relation to the process variable. The empirical relationship between the hydrogen yield and pH, microwave duration,

Y ¼ 0.61 þ 0.17A 0.11B 0.031C 0.078AB þ 0.038AC
0.040BC þ $0.12A^2$ $0.088B^2$ $0.10C^2$ (4)

Where Y represents hydrogen molar yield, A, B, and C rep- resents pH, microwave duration and microwave intensity respectively.

The regression equation was subjected to analysis of variance (ANOVA) in order to evaluate its statistical signifi- cance. The summary of the analysis of variance of the model is presented in Table 2, with the high F value (5.47) and P value lower than 0.05 demonstrating the significance of the model [34]. However, some model terms included in the Equation (4) were shown as insignificant (P > 0.1, Table 2). The coefficients of estimates and their confidence intervals are shown in Table

3. The obtained model had a coefficient of determination

3.2. Interactive effects of the treatment parameters inoculum efficiency

The three dimensional response surface curve and the cor-

responding contour map plots showing the interaction among the parameters pair wise are presented in Fig. 1AeF. The fig- ures represent the relative effects of two variables within the experimental range on hydrogen yield with the third variable maintained at its median value. The interactions between pH and microwave duration are shown in Fig. 1A and the corre- sponding contour plot (Fig. 1B). The response surface with a clear peak suggests that the maximum hydrogen yield could be achieved within the design boundary. The hydrogen molar yield increased as the

Table 2 e Analysis of variance generated from the Box-Behnken design.

Source	Sum of	df	Mean of	F-value	P-value	R^2
Model	0.50	9	0.055	5.47	0.017	0.8756
A	0.23	1	0.23	22.84	0.002	
B	0.095	1	0.095	9.35	0.01	
C	0.0007	1	0.0007	0.77	0.40	
AB	0.024	1	2.37	0.16	0.16	
AC	0.0005	1	0.0005	0.56	0.48	

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

Maximum hydrogen yield was observed at inoculum pre- treatment with pH 11 and microwave treatment duration of

2 min. This could be a result of the joint inhibitory effect of pH and microwave treatment given that the methanogens pre- sent in the inoculum will be suppressed at such extreme pH values. Furthermore, this combination treatment will also enrich the hydrogen producing bacteria such as Clostridium spp. as they are more suited to survive in harsh conditions due to the inherently higher stress resistance of endospores [35]. This is similar with the result reported by Cai et al. [36] who observed a maximum hydrogen yield with alkaline pre- treatment at pH 11.

The interaction between pH and microwave intensity on the process yield is shown in Fig. 1CeD. The hydrogen molar yield increased with increase in pH and microwave intensity up to 11.6 and 850 W respectively. A maximum hydrogen yield of 0.8 mol H_2/mol glucose was observed at inoculum pre- treatment with pH 11.6 and microwave intensity of 850 W. Above these values, hydrogen yield rapidly decreased, this observation is expected as the

Table 3 e Coefficients of estimates of the variables and their confidence intervals.

Component	Coefficient	df	Standard	95% CI	95% CI
Intercept	0.61	1	0.045	0.50	0.71
A	0.17	1	0.036	0.086	0.25
B	0.11	1	0.036	0.19	0.025
C	0.031	1	0.036	0.12	0.053
AB	0.078	1	0.050	0.20	0.041
B^2	0.088	1	0.049	0.027	0.20
AC	0.038	1	0.050	0.081	0.16

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

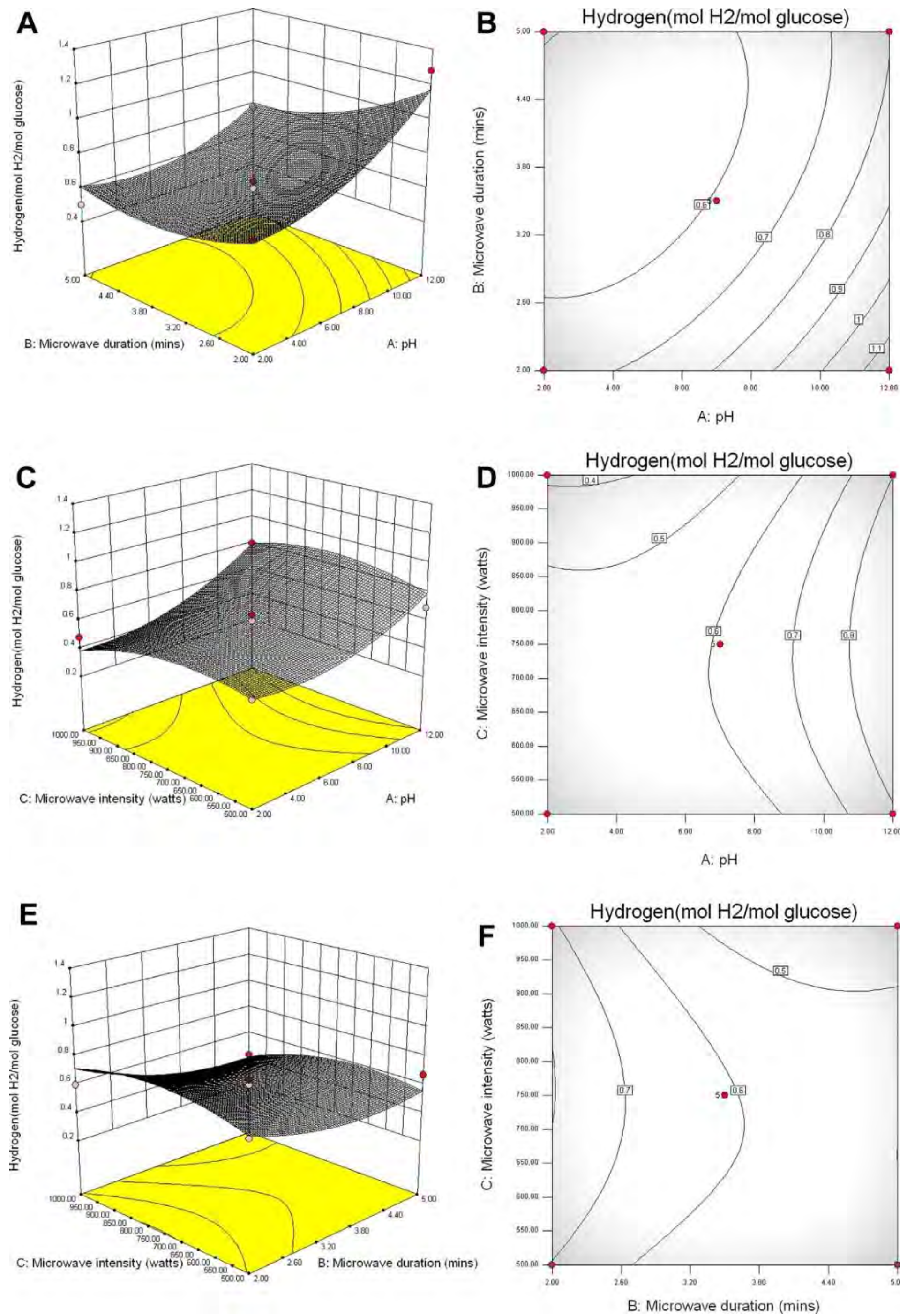

Fig. 1 e A: Response surface plot showing the interaction between pH and microwave duration. B: Contour map plot showing the interaction between pH and microwave duration. C: Response surface plot showing the interaction between pH and microwave intensity. D: Contour map plot showing the interaction between pH and microwave intensity. E: Response surface plot showing the interaction between microwave duration and microwave intensity. F: Contour map

shock pre-treated sludge only contained endospore forming hydrogen producing bacteria which will be mainly Clostridium spp [37] while microwave treatment has been employed suc- cessfully to inactivate members of the family Enter-

Similarly, the interactions between microwave duration and microwave intensity showed a significant impact on hydrogen yield. As observed in Fig. 1EeF, hydrogen yield increased with increase in microwave intensity and micro-

2 min respectively. Further increase led to a decrease in

hydrogen yield from 0.7 mol H_2/mol glucose to 0.6 mol H_2/mol glucose. The optimum conditions were observed to be 790 W and 2 min for microwave intensity and duration respectively. To further understand the mechanism of this hybrid treat- ment, the composition of the optimized pre-treated inoculum was analyzed and presented in Table 4.

During the pre-treatment, the temperature of the raw sludge increased from 11 C to 81 C suggesting the release of heat energy into the inoculum. Microwave irradiation can produce rapid and more uniform heating with less energy consumption than conventional heating [39]. The resulting heat treatment from the microwave can effectively inhibit the activity of the non spore forming hydrogenotrophic metha- nogens and enrich the endospore forming hydrogen produc- ing bacteria to improve biohydrogen production.

Microwave treatment can also increase the biodegradability of anaerobic sludge by solubilizing the organic matter in the sludge. The soluble COD of the raw sludge increased from

11.20 g/l to 27.01 g/l (Table 4) after pre-treatment indicating that the treatment enhanced the sludge particle disintegration. Park et al. [40] and Rani et al. [41] investigated the effects of secondary sludge pre-treatment by microwave irradiation and reported a 22% and 20% increase in sCOD after pre-treatment. A similar observation was reported by Thungklin et al. [25] for hydrogen production using microwave pre-treated poultry slaughter house sludge in which the soluble COD increased from 15.34 g/l to 25.79 g/l after pre-treatment.

Microwave treatment can effectively disrupt the complex sludge floc structure and release extracellular and intracel- lular biopolymers such as proteins, carbohydrates and lipids into a soluble form thereby improving the solubilization of particulate organic matter

3.3. Optimization of the hybrid technique for inoculum pre-

The regression equation was solved by the method of Myers

Table 4 e Characteristics of anaerobic sludge prior to and after pre-treatment.		
Parameters	Raw sludge	Optimized pre-treated sludge
pH	7.35	11.02
VSS(g/L)	10.23	34.15
Temperature (C)	11	81

points for inoculum development. The predicted optimal pre-

treatment set points values were pH 11, 2 min microwave treatment and 860 W microwave intensity with a biohydrogen yield of 1.45 mol H_2/mol glucose. Validation experiments using the predicted optimal conditions were carried out in replicates and yielded 1.92 mol H_2/mol

3.4. Biohydrogen trend and metabolites produced during the semi pilot fermentation

The pilot scale-up fermentation process was operated in a

batch system for 96 h using the optimized pre-treated inoc- ulum. Knowledge of the process dynamics at this scale is essential for an efficient industrial biohydrogen production. Two semi pilot scale processes were carried out under the same optimal conditions, but with the difference in the pH control state. The rationale was that a high yield and stable production of hydrogen in a dark

3.4.1. Pilot process without pH feedback control

Hydrogen gas evolution started from the 19th hour of the fermentation, which indicates a long lag phase. This may be a result of the time needed for the microorganisms to adjust to the new environment, and synthesize enzymes needed for growth and metabolism. Zhou et al. [45] reported a long lag phase of 11 h during fermentative codigestion of food waste with anaerobic sludge for biohydrogen production. Heat treated inocula contains endospores that will require specific nutrients and physicochemical conditions to induce germi- nation to form vegetative cells which in turn are then able to grow and increase the metabolically active biomass [46].

In addition, exposure to oxygen during the inoculum transfer may have an inhibitory effect on the hydrogen pro- ducing ability of such microorganisms. A lag phase of 50 h was observed for hydrogen production with sucrose and heat treated compost inoculum [47]. The predominant bio- hydrogen producers Clostridium spp. are strictly anaerobic microorganisms [48] therefore reactor design has to take into account the need for anoxic conditions although this may be difficult considering the size of large scale operations [49].

A similar lag phase of 20 h has been reported by Logan et al. [50] in a fermentative hydrogen production experiment using heat shocked soil inoculum. Process parameters such as reactor configuration and volume size can affect the partial pressure and heat transfer during fermentation in large scale processes which in turn may lead to a longer lag phase [51]. Hydrogen production systems should be designed and oper- ated in a way that the concentration of hydrogen in the reactor is reduced to prevent the feedback inhibition [4]. Studies have reported different methods of reducing the hydrogen partial pressure which include gas sparging [52] and hydrogen removal by vacuum [53]. Recently, an energy saving separation method was

observed after 70 h of fermentation (Fig. 2), corresponding to a

hydrogen yield of 1.78 mol H_2/mol glucose.

The initial pH decreased from 6.0 to 5.63 at the start of hydrogen production (20 h) and gradually dropped to 4.37 at

70 h which is due to the production of volatile fatty acids by the hydrogen producing bacteria. Clostridia produce VFAs and hydrogen in the exponential growth phase and switch to rapid alcohol production in the late growth phase [55]. Hydrogen production lasted up to 71 h after which a rapid decline in hydrogen concentration was observed. This reduction in hydrogen production could be attributed to the low pH (4.37) in the culture medium. The ability to produce hydrogen by the endospore forming bacteria is governed by the pH, buffering capacity of the medium as well as the nature of the carbon source [56]. For instance, hydrogen producing bacteria such as Clostridium pasteurianum do not grow well at low pH and their metabolism will be shifted away from hydrogen production to solvent production [55].

The time course of hydrogen production; glucose utilization and pH evolution are shown in Fig. 2. The metabolites produced were acetate (35.48%), butyrate (63.5%) and propio- nate (1.02%). As expected on microbiological

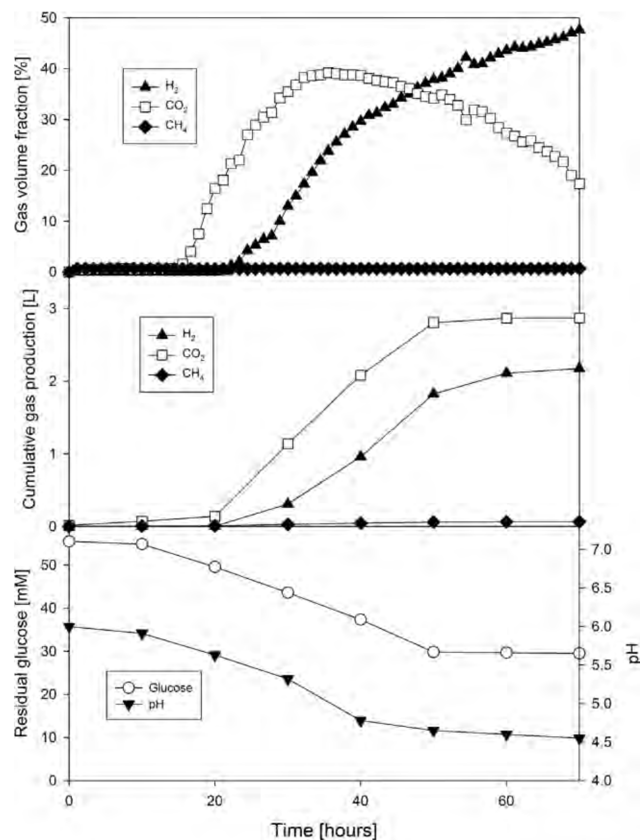

Fig. 2 e Time course of hydrogen production for pilot scale-up process without pH control.

process inhibition [50], this was observed in our study as only

46% of substrate was utilized which could be a result of the accumulation of volatile fatty acids inhibiting the metabolic activity of the hydrogen producing bacteria.

Fang and Liu [57] reported a decrease in glucose utilization from 100% at pH 6e45% at pH 4; they concluded that these effects were caused by process inhibition by the movement of the non-dissociated acids through the permeable cellular membrane. The non-dissociated acids once inside the cell will dissociate to release a proton due to the higher intracellular pH. Therefore, in order to maintain the intracellular pH, more metabolic energy is needed to

3.4.2. Pilot process with a controlled pH state

In this semi pilot scale batch, the pH was controlled and maintained at 6.0 throughout the fermentation using the bioreactor feedback control loop. Hydrogen production star- ted at the 7th hour of fermentation and rapidly increased up till the 60th hour. A shorter lag phase was observed in this batch compared to the previous batch; this may be a result of the influence of pH control. A peak hydrogen concentration of

56.8% was observed at 59thhour after which a rapid decline in hydrogen concentration was observed.

A cumulative hydrogen production of about 11.01 L was observed within 60 h of fermentation corresponding to a hydrogen yield of 2.07 mol H_2/mol glucose. The summary of the pH controlled pilot process is presented in Fig. 3. Lin et al. [59] reported maximum hydrogen yield of 2.34 mol H_2/mol sucrose in a pilot scale hydrogen production with a working volume of 400 L and a maximum hydrogen concentration of

35.8% during the continuous process. The variation in the result may be due to differences in the substrate used, mode of operation, working volume, and inoculum pre-treatment method.

pH plays a vital role during hydrogen production by gov- erning the metabolic activity of the hydrogen producing

3.5. Effect of pre-treatment on microbial

Viable microbial counts were established to further investi-

gate the influence of the pre-treatment method during fermentation on the presence of well-known hydrogen pro- ducing microbes such as Clostridium spp. Presumptive Clos- tridium spp. counts increased slightly from initially about

2.8 10^5 CFU/mL to about 2 10^9 CFU/mL by the 36th hour of

the fermentation (Fig. 3). The growth continued rapidly as the fermentation progressed to reach a peak after 48 h for the anaerobe and clostridial count of about 5 and 7 10^9 CFU/mL. In addition, the observed increase in the presumptive Clos- tridium spp. counts over time resulted in

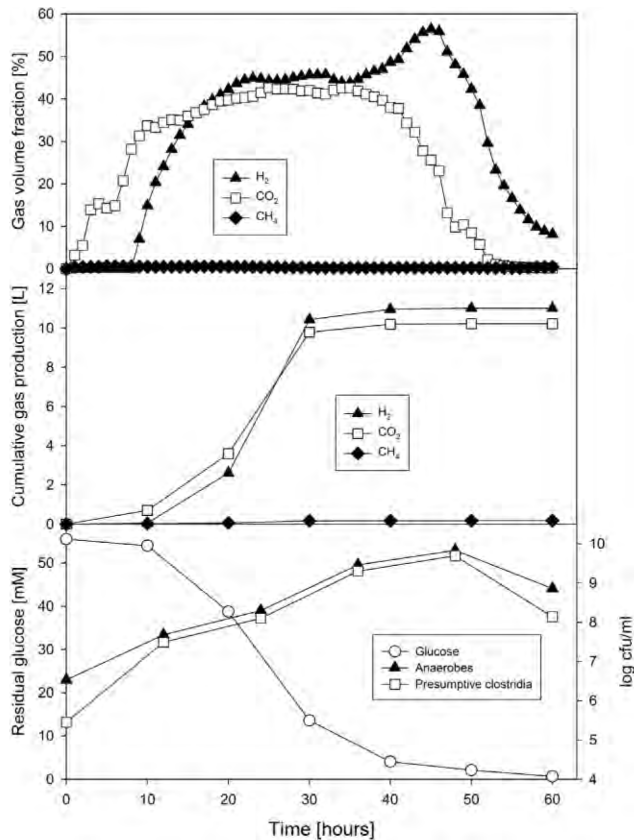

Fig. 3 e Hydrogen production performance in pilot scale-up process with pH control.

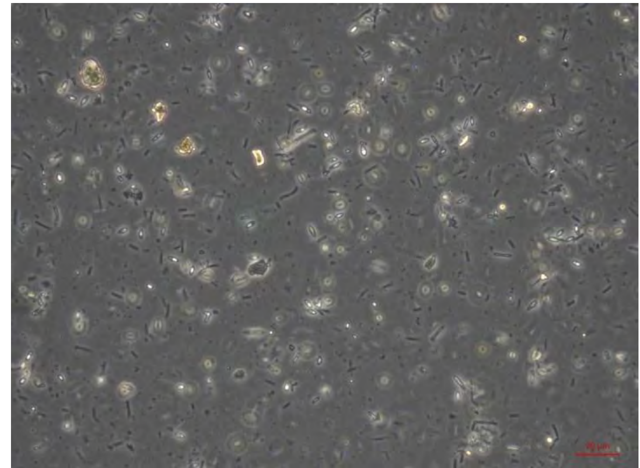

Fig. 4 e Presence of endospore forming presumptive clostridia in a reactor sample taken after 24 h demonstrated by phase contrast microscopy.

Thereafter, hydrogen concentration gradually increased to 56.43% at the 48th hour with a corresponding increase in the counts to about 5 10^9 CFU/mL. All randomly selected PCA and DRCM isolates were Gram positive, catalase and oxidase negative, straight rods occurring singly or in pairs. They were motile and formed either central or terminal endospores. The above characteristics are typical features of species within the genus Clostridium [31,61]. These results indicate the presence of Clostridium spp., which was also confirmed by phase contrast microscopy of the process samples over time (Fig. 4). Thus, the pre-treatment method successfully enriched for endospore forming hydrogen producing bacteria. Although we have not taxonomically confirmed the identity of pre- sumptive clostridial isolates at species level, it is known that species such as Clostridium pasteurianum, C. acetobutylicum, and C. butyricum produce acid and hydrogen via fermentation of carbohydrates to

4. Conclusion

The effect of combined pH and microwave inoculum pre-treatment on biohydrogen production was modeled and optimized using the response surface methodology. The op- timum conditions predicted by the model were 11, 2 min and 860 W for pH, microwave duration and microwave intensity respectively. A maximum hydrogen molar yield of 1.92 mol H_2/mol glucose was achieved in the validation experiment corresponding to a 32.41% increase in hydrogen yield. Methane production was effectively suppressed indicating the effectiveness of this combined pre-treatment method to enrich hydrogen producing bacteria. Furthermore, this tech- nique presents a low cost and energy saving approach as evident from the short microwave treatment duration compared to conventional heating which has been widely used.

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Two semi pilot scale-up processes were carried out with the optimal pre-treated inoculum to study the process dy- namics and the influence of pH control. A maximum hydrogen molar yield of 1.78 mol H_2/mol glucose was observed in the absence of pH control compared with 2.07 mol H_2/mol glucose in the pH controlled process. These results indicate that pH control enhanced fermentative hydrogen production significantly. While this hybrid pre-treatment via pH and mi- crowave can be encouraged for fermentative hydrogen inoc- ulum development, a thorough assessment of energy gain should be considered.

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CHAPTER 5

Optimization of fermentative hydrogen production from potato waste using Artificial Neural Network and Response surface methodology-preliminary scale up

This chapter has been submitted for publication in a peer review journal with the title: Optimization of fermentative hydrogen production from potato waste using Artificial Neural Network and Response surface methodology-Preliminary scale up. The manuscript is presented in the following pages.

However additional data not included in the manuscript are presented in an appendix following this chapter.

Highlights

- Potential of biohydrogen production from potato peels waste was evaluated
- ANN and RSM techniques for bioprocesses modelling were comparatively assessed in fermentative hydrogen production
- ANN showed a better predicting ability with a percentage error 7.65% against 82.72% for RSM
- The semi pilot scale process gave a hydrogen yield of 239.94mL/g TVS.

Optimization of fermentative hydrogen production from potato wastes using Artificial Neural Network and Response surface methodology- Preliminary scale up

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ABSTRACT

The study comparatively modeled and optimized biohydrogen production from potato wastes using Artificial Neural Network (ANN) and Response surface methodology (RSM). The input factors considered were nutrients supplementation (%), substrate concentration (g L^{-1}), pH and temperature (°C). The ANN model consisted of a committee of networks with a topology of 5-(4, 6, 7, 9, 10)-1 structured on a back propagation architecture. Coefficient of determination (R^2) of 0.99 and 0.85 were obtained with ANN and RSM respectively.

Experimental validation of the predicted optimal process conditions gave a hydrogen yield of 186.72 mL g^{-1} TVS and 159.32 mL g^{-1} TVS with a prediction error of 7.65% and 82.72% for ANN and RSM respectively. A semi pilot process under the optimized condition gave a maximum hydrogen yield of 239.94 mL g^{-1}TVS. These results highlights the modelling efficiency of ANN on complex non linear bioprocesses and the prospect of scaling up biohydrogen production from potato peels.

Keywords: Biohydrogen production, Response surface methodology, Artificial neural network, Potato waste, Genetic Algorithm.

1. INTRODUCTION

Hydrogen has emerged as one of the most promising, environmentally friendly renewable energy sources. Its specific enthalpy (141.9kJlg) is almost three times higher than that of conventional hydrocarbon fuels such as gasoline (47.9KJ/g). At the same time water is the only product of combustion therewith rendering hydrogen as an attractive and clean fuel (Kotay and Das, 2008). Currently about 88% of commercial hydrogen is produced from fossil fuels such as natural gas, heavy oil and coal while about 4% stems from the electrolytic cleavage of water (Nath and Das, 2003). However, these techniques are not sustainable and highly energy intensive which necessitates the need to optimize fermentative hydrogen production from low cost substrates.

Agricultural residues and food waste are abundant, cheap and readily available organic substrates (Guo *et al*., 2010). Food waste is a potential substrate for fermentative hydrogen production due to its content of ready degradable carbohydrates, proteins and fats (Yuan *et al.,* 2006). All of these can be utilized as substrates by *Clostridium* spp. with carbohydrates remaining the preferred substrates for hydrogen production (Nandi and Sengputa, 1998).

In 2011, South Africa alone generated approximately 5.9×10^7 tonnes of general waste with food waste accounting for about 34% of the total waste of which zero percent was recycled (DEA, 2012). Waste management in South Africa is therefore still largely dependent on disposal in landfills (DEA, 2012). Food waste disposal in landfills without proper management is undesirable for hygiene reasons (rodents) as it can cause air pollution (malodour, release of microorganisms) and ground-water contamination (Cantrell *et al.*, 2008).

Potatoes (*Solanum tuberosum*) are mostly used directly for human consumption but they are also processed into a variety of convenience products such as mashed potatoes or chips. During processing, 20-50% of the raw products with 2.25×10^6 tonnes of potatoes produced

in South Africa in 2012 (DAFF, 2013) are transformed into starch containing wastes such as peels.

Biohydrogen production from organic matter is highly influenced by environmental factors such as temperature, pH, nutrient availability and process parameters such as reactor configuration and substrate concentration (Li and Fang, 2007). In order to maximise hydrogen yields using dark fermentation, these factors needs to be optimized by the development of an accurate process model based on the key operational conditions to

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is laborious, time consuming and fails to consider the interactive relationship between different process parameters (Pan *et al*., 2008).

These limitations have been overcome by using more efficient design of experiment methods such as Response Surface Methodology (RSM) and Artificial Neural Network (ANN). RSM is a collection of statistical techniques for the design of experiments, to build models and evaluate the effect of process factors in order to find the optimum conditions. It involves the design of statistical experimental procedures in which several factors are simultaneously varied (Kalil *et al*., 2000).

Artificial neural network (ANN) has emerged as an attractive tool for non-linear multivariate modelling. It is generic in structure and possesses a high capacity to learn from historical data. It has the merits of simplicity of simulation, prediction and modelling with fewer requirements for mathematical description (Desai *et al.,* 2004). ANNs are efficient in developing bioprocess models without a prior understanding of the kinetics of metabolic fluxes within the cell and the cultural environment; it understands and computes the relationship between input and output variables in a similar pattern like the human brain (Whiteman and Gueguim Kana, 2014).

Modelling by ANN can be optimized using Genetic Algorithm (GA). GA is an artificial intelligence based optimization technique that mimics the principle of biological evolution based on the survival of the fittest and random data exchange of chromosome during propagation resulting to new species (Haider *et al*., 2008). RSM and ANN have been reported in bioprocess modelling and optimization including biohydrogen production (Wang and Wan, 2009; Nasr *et al*., 2013), but to the best of our knowledge a comparative optimization of hydrogen production from potato wastes using RSM and ANN has not yet been reported.

This study modelled and optimized biohydrogen production from potato peels waste on the input parameters of temperature, pH, substrate concentration and nutrients supplementation by the application of RSM and ANN coupled with Genetic Algorithm. Furthermore, a preliminary assessment of the pilot scale up production was carried out to examine the applicability of this low cost feedstock for future large scale biohydrogen production.

2. Materials and Methods

2.1. Inoculum

Anaerobic sludge used as the inoculum in this study was obtained from the Darvill wastewater plant, Pietermaritzburg, South Africa. Prior to use, the sludge was filtered through a 20 mesh sieve to remove large solid particles. A hybrid pre-treatment method of pH and autoclave as described by Faloye *et al*. (2013) was used to deactivate the hydrogen consuming methanogens and to enrich endospore forming hydrogen producing bacteria.

2.2. Feedstock and pre-treatment

Potato peel wastes were collected from a local restaurant in Pietermaritzburg, South Africa. The peels were sorted and dried at 60°C overnight and reduced in particle size by milling to pass a 20 mesh screen. The grounded potato peels waste (concentration specified in the experimental design) was pre-treated by boiling with sterile distilled water at 100°C for 30 min to release the starch. The composition of the dried potato peels waste is listed as follows:

Total solids (TS) – 90.72%, Total volatile solids (TVS) – 80.24%, Total starch – 42.47%, and Total sugars – 48.71%.

The nutrients supplement comprised of (g L^{-1}): KH_2PO_4 - 1.5, $FeCl_2$ - 0.1, $CaCl_2$. $2H_2O$ - 0.1, $MnCl_2$. $6H_2O$ - 0.1, $ZnSO_4$ - 0.05, Na_2MoO_4 - 0.01, $NaHCO_3$ - 4 and was sterilized at 121°C for 15min.

2.3. Experimental design

A four factor Box-Behnken design was used to generate twenty six fermentation batches with varied input conditions. The four independent variables of nutrients supplementation (%), substrate concentration (g L^{-1}), pH and temperature (°C) were considered as input parameters. The search range and the levels of the parameters are shown in Table 1 and the centre points were replicated five times to estimate the experimental errors.

Table 1: Experimental design with parameter ranges

Input Parameter	Code	Input Range	Coded values
Nutrients supplement (%)	A	0-100	0, 50, 100
Substrate concentration (g L^{-1})	B	10-50	10, 30, 50
pH	C	5-8	5, 6.5, 8
Temperature(°C)	D	30-55	30, 42.5, 55

2.4. Batch fermentation process

Fermentation experiments were carried out in modified 250 mL Erlenmeyer flasks. Each flask was inoculated with 10% (v/v) pre-treated sewage sludge then fed with treated potato peel waste, nutrients supplement according to the experimental design and made up to a total volume of 200 mL with sterile distilled water. The control set points of pH and temperature were maintained as specified in the experimental design (Table 2). The initial pH was adjusted by adding 1M HCl or 1M NaOH solution and the flasks were flushed for 3min with nitrogen to provide anaerobic conditions and capped tightly with rubber stoppers. The process time was set to 72h and the twenty six fermentation batches were carried out in triplicate.

2.5. Analytical methods

Hydrogen, carbondioxide and methane concentrations as well as the gas volume were determined as reported previously (Faloye *et al*., 2013). The cumulative volume of hydrogen produced was calculated according to Equation 1:

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

$V_{H,i}$ and $V_{H,\,i-1}$ are cumulative hydrogen gas volume at the current (i) and previous (i-1) time intervals, $V_{G,i}$ and $V_{G,i-1}$ the total biogas volumes in the current and previous time intervals, $C_{H,i}$ and $C_{H,i-1}$ the fraction of hydrogen gas in the current and previous time intervals, and V_H the total volume of headspace in the reactor. Hydrogen yield was calculated by dividing the cumulative hydrogen produced by the total volatile solids (TVS) of the substrates added. pH was measured by using a calibrated pH meter (Crison, South Africa). The concentrations of total solids (TS) and Total volatile solids (TVS) were determined according to standard methods (APHA, 2005). Total sugar and Total starch were determined by the colorimetric method of Dubois *et al.* (1956) and the method of AOAC (1990) respectively.

Table 2: Box-Behnken design and their corresponding hydrogen yields

Run	Nutrients supplement (%)	Potato concentration(g/L)	Initial pH	Temperature(°C)	Hydrogen yield(mL/g TVS)		
					Experimental	RSM predicted	ANN predicted
1	0	30	8.0	42.5	47.57	26.31	34.32
2	100	30	6.5	30	180.58	158.93	171.91
3	50	10	6.5	30	112.83	89.33	102.25
4	0	50	6.5	42.5	165.81	142.48	165.89
5	100	30	6.5	55	0.06	3.09	3.70
6	50	50	8.0	42.5	0.05	33.98	9.66
7	50	30	5.0	30	73.31	108.55	72.52
8	50	50	5.0	42.5	0.37	33.90	6.75
9	50	30	6.5	42.5	14.45	19.84	14.54
10	50	30	8.0	30	34.94	89.66	43.93
11	50	10	6.5	55	0.22	35.82	4.34
12	0	10	6.5	42.5	1.61	3.00	7.55

13	50	50	6.5	55	0.31	5.76	1.72
14	0	30	6.5	55	0.13	35.11	4.98
15	100	50	6.5	42.5	38.47	42.51	30.24
16	0	30	5.0	42.5	0.07	-1.30	-3.58
17	50	30	5.0	55	0.07	- 49.21	-9.17
18	50	10	8.0	42.5	0.18	-20.75	5.05
19	100	10	6.5	42.5	43.25	72.01	43.63
20	50	50	6.5	30	283.01	229.36	271.42
21	100	30	8.0	42.5	0.21	-16.46	-4.16
22	50	30	6.5	42.5	25.23	19.84	14.54
23	50	10	5.0	42.5	0.02	-21.31	1.80
24	100	30	5.0	42.5	7.31	10.51	12.10
25	50	30	8.0	55	0.13	-29.67	-4.44
26	0	30	6.5	30	147.56	157.12	150.26

2.6. Response surface methodology

Experimental data obtained from the twenty six fermentation batches were fitted to a second order polynomial model (Eq. 2) which relates the independent variables with the predicted hydrogen yield.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i \qquad (2)$$

Where Y is the predicted response (hydrogen yield); β_0 a constant; β_i the linear coefficient; β_{ii} the squared coefficients and β_{ij} the cross product coefficients; and X_i and X_j the input variables.

The fitness of the model was estimated by the Analysis of Variance (ANOVA) using Design-Expert 8 software (Stat-Ease, Inc. USA). The quadratic equation obtained from the model was solved by the method of Myers and Montgomery (1995) to obtain the predicted optimum process conditions of hydrogen production for the RSM.

2.7. ANN structure and training

A feed forward neural network with multilayer perceptron (MLP) architecture was structured. A committee made up of five ANNs as depicted in Figure 1 was implemented in a PHP scripting environment. A topology of 5-(4, 6, 7, 9, 10)-1 was adopted which corresponded to the number of neurons of inputs, hidden (variables) and output layers. This architecture had a feed forward nature in which the neurons present in the input layer fed the neurons within the hidden layer using a scheme of adjustable weights (Desai *et al.,* 2008).

A single layer configuration was selected for the hidden layer with a sigmoid transfer function. The input layer consisted of five neurons (nutrients supplementation, substrate concentration, initial pH, temperature and bias) while the output layer was the hydrogen yield response. The functional relationship estimated by the ANN model can be represented with Eq. (3)

$$Y = f(X_1, X_2, X_3, X_4) \qquad (3)$$

Where X_1, X_2, X_3 and X_4 represents the independent variables (nutrients supplement, substrate concentration, initial pH, temperature) and Y represents the output (hydrogen yield). The network was trained using the Levenberg-Marquardt Algorithm or Back propagation method (Costa *et al.,* 2007) in which the weight connections between the neurons were adjusted in order to minimize the error difference (root mean square error (MSE)) between the predicted and the experimental outputs below an acceptable threshold according to Equation 4:

$$\textbf{RMSE}=\sqrt{\frac{\sum_{i=1}^{N}\sum_{n=1}^{M}\left(y_n^i-\hat{y}_n^i\right)^2}{NM}} \qquad (4)$$

Where N is the number of patterns used in the training, M is the number of output nodes, i is the index of the input pattern (vector) and y_n^i and $\hat{y}_n^i$ are the actual and predicted outputs. Eighty percent of the experimental data were used to train the ANN model while the remaining twenty percent were kept for model validation. Model validation was carried out by predicting the hydrogen yield on data points that were not previously used for training; the predicted and experimental values were then compared.

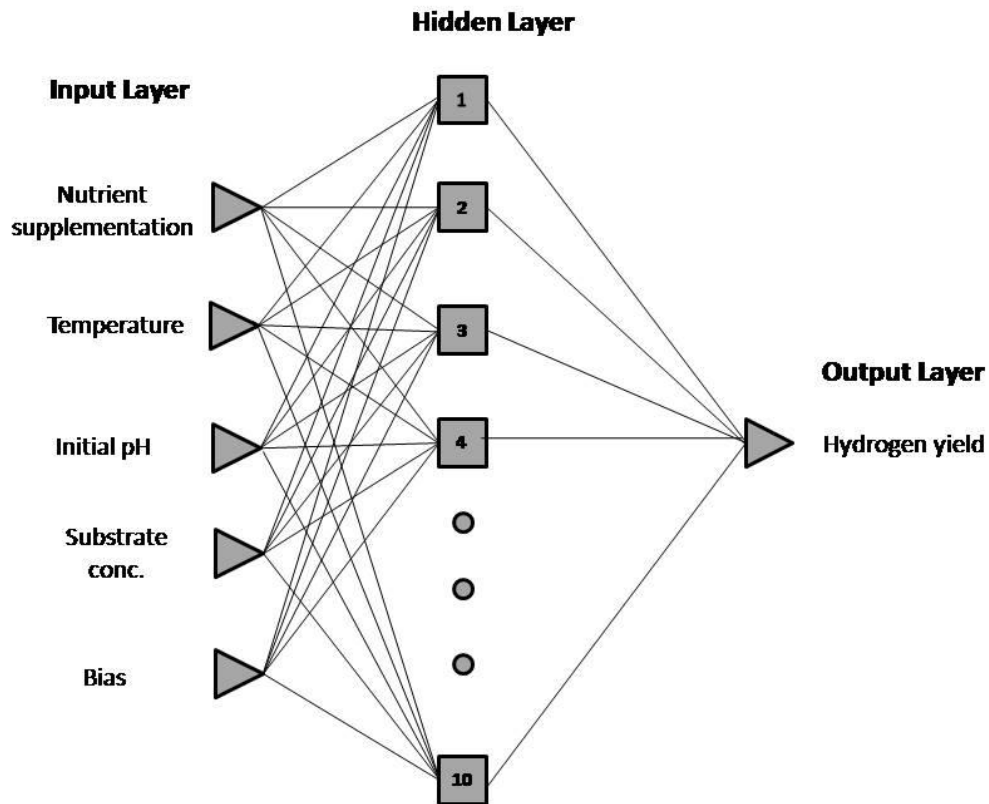


Figure 1: Schematic representation of ANN structure with one input layer (five neurons), one hidden layer and one output layer.

2.8. Hydrogen yield optimization using Genetic Algorithm

The optimum operational process parameters based on the ANN model were determined using genetic algorithm running on Biopro-optimizer software (Gueguim Kana *et* al., 2010). Each run in the experimental data represented a chromosome containing four genes which were nutrients supplement (%), substrate concentration (g L^{-1}), pH, and temperature (°C). The GA settings of population size, parent size, crossover and mutation rate used were 26, 60%, 10% and 30% respectively. The fitness of each chromosome for hydrogen yield was assessed using the developed ANN committee model which served as the objective function. The best chromosomes were selected and replicated using GA operators namely mutations and cross-overs to produce the next generation. The average performance increased from one generation to another. The cycle was repeated until the stopping criterion was met. The optimum process conditions determined by both ANN-coupled GA and the RSM were validated experimentally. The percentage error between the experimental and the predicted values were computed according to Equation 5:

$$\text{Percentage error} = \frac{\text{Experimental value} - \text{Predicted value}}{\text{Experimental value}} \times 100 \qquad (5)$$

2.9. Hydrogen production in a semi-pilot reactor

2.9.1. Pilot scale fermentation process

The validated optimum process conditions based on ANN were used for the preliminary pilot scale up process. An initial fermentation stage was carried out in a 1L modified Erlenmeyer flask bioreactor with a total volume of 700 mL to seed the semi pilot bioreactor. The flask was inoculated with 10% (v/v) of the pre-treated anaerobic sludge and fed with 50g/L of substrate concentration and 10% nutrient supplementation. The optimal physico-chemical parameters validated earlier were maintained and this stage was carried out for 24h.

The pilot scale fermentation process was carried out in a 10L bioreactor (Labfors Infors HT bioreactor, Switzerland). The reactor was sterilized prior to its use by autoclaving at 121°C for 15min. It was fed with the optimum concentrations of potato waste and nutrients supplement to a working volume of 7L and then inoculated at 10% (v/v) with the 24h old active culture from the previous stage. The reactor was flushed with nitrogen gas for 5min to ensure anoxic conditions. The operational set points of pH, temperature, agitation rate and process time of 6, 30°C, 150 rpm and 72h respectively were maintained throughout the fermentation process.

2.9.2. Pilot scale process monitoring and analysis

The fractions of the gas evolving from the process were continuously monitored through an array of sensors (BCP-H_2, BCP-CH_4, BCP-CO_2) (Bluesens, Germany) and the biogas volume was monitored using a milligas counter (MGC, Bluesens, Germany) as reported previously(Faloye *et al*., 2014). These sensors were interfaced to the Flab-Biogas software (Gueguim Kana *et al*., 2013) running at 1min sampling frequency. The cumulative volume of hydrogen, methane and carbon dioxide was recursively software computed using the different fractions in the evolving gas and the gas volume at each sampling interval according to Eq. (1).

The volatile fatty acids (VFAs) acetate, butyrate, propionate, valerate and isovalerate were determined by gas chromatograph via flame ionization detection as reported previously (Faloye *et al*., 2013).

3. Results and discussion

3.1. Response surface model analysis

The result of the Analysis of variance (ANOVA) of the quadratic model is summarized in Table 3. The ANOVA of the RSM model showed a P value of less than 0.05 and an F value of 4.64 which indicates the significance of the model. A high coefficient of determination

(R^2- 0.85) demonstrated the good correlation between the model prediction and the actual response. Thus 85% variations in the response can be explained by the model as R^2 values greater than 0.7 are considered significant (Desai *et al*., 2008).

The adequate precision measures the signal to noise ratio with a ratio greater than 4 showing the appropriateness of the model. In this study, a ratio of 8.71 was obtained indicating an adequate signal; therefore the obtained model was used to navigate the design space.

As shown in Table 3, the linear terms of substrate concentration (B), temperature (D), and the square term of temperature (D^2) were highly significant with P values of less than 0.05. This indicates that these terms greatly affected the hydrogen yield.

The model can be mathematically expressed according to Eq. (6)

$$Y = 19.84 - 7.74A + 27.49B + 0.16C - 69.28D - 42.25AB - 13.65AC - 8.27AD - 0.12BC - 42.52BD + 9.61CD + 21.74A^2 + 23.43B^2 - 36.81C^2 + 46.80D^2 \quad (6)$$

Where Y represents hydrogen yield and A, B, C and D represents nutrients supplementation, substrate concentration, initial pH, and temperature respectively.

The optimum operational conditions for maximum hydrogen yield predicted by the RSM model were 1.03% of nutrients supplementation, 50g L^{-1} substrate concentration, initial pH of 6.53 and fermentation temperature of 30°C.

Table 3: Analysis of variance of the RSM model

Source	Sum of Squares	df	Mean of Squares	F-Value	P-value
Model	0.00001150	14	8213.86	4.64	0.0073
A	718.74	1	718.74	0.41	0.5370
B	9070.05	1	9070.05	5.12	0.0448
C	0.31	1	0.31	0.000175	0.9897
D	57589.69	1	57589.69	32.54	0.0001
AB	7138.56	1	7138.56	4.03	0.0698
AC	745.29	1	745.29	0.42	0.5297
AD	273.74	1	273.74	0.15	0.7016
BC	0.058	1	0.058	-0.0000325	0.9956
BD	7232.65	1	7232.65	4.09	0.0682
CD	369.22	1	369.22	0.21	0.6567
A^2	2062.22	1	2062.22	1.17	0.3035
B^2	2394.80	1	2394.80	1.35	0.2693
C^2	5912.89	1	5912.89	3.34	0.0948
D^2	9559.11	1	9559.11	5.40	0.0403

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

Table 4: Coefficients of estimates of the variables and their confidence intervals

Component	Coefficient estimate	df	Standard error	95% CI Low	95% CI High
Intercept	19.84	1	29.75	-45.63	85.31
A	-7.74	1	12.14	-34.47	18.99
B	27.49	1	12.14	0.76	54.22
C	0.16	1	12.14	-26.57	26.89
D	-69.28	1	12.14	-96.01	-42.55
AB	-42.25	1	21.03	-88.54	4.05
AC	-13.65	1	21.03	-59.95	32.65
AD	-8.27	1	21.03	-54.57	38.02
BC	-0.12	1	21.03	-46.42	46.18
BD	-42.52	1	21.03	-88.82	3.77
CD	9.61	1	21.03	-36.69	55.90
A^2	21.74	1	20.14	-22.59	66.06
B^2	23.43	1	20.14	-20.90	67.75
C^2	-36.81	1	20.14	-81.14	7.51
D^2	46.80	1	20.14	2.48	91.13

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

3.2. Influence of the process parameters on hydrogen production

Environmental factors such as initial pH, temperature and substrate concentration are crucial in fermentative hydrogen production. These factors greatly influence the growth and metabolic activity of hydrogen producing bacteria which in turn impacts the production efficiency.

The three dimensional response surface curves (Figure 2A-2F) represent the interactive effects of pair wise process parameters on hydrogen yield while keeping the remaining parameters at their median value.

Nutrients supplementation

Nutrient supplementation can increase hydrogen yields by enhancing the growth and activity of hydrogen producing microorganism (Lin and Lay, 2005). The interactive effects of nutrient supplementation and substrate concentration, initial pH, and temperature are shown in Figures 2A-2C. The response curves have a clear peak which suggests that the optimum conditions could be found within the design boundaries. As shown in Fig. 2A, nutrients supplement in excess of 10% decreased hydrogen yields from 120mL/g TVS to 20mL/g TVS. A maximum hydrogen yield of 120 mL/g TVS was obtained with 10% nutrients supplementation and 50g/L substrate.

Essential macro and micro elements such as N, P, S, Mg and Fe are important for biomass assimilation and pH stabilization. They are also essential for the activity of ferredoxins and hydrogenases which in turn are crucial for hydrogen production (Das and Veziroglu, 2001). However, exceedingly high concentrations of these elements may trigger reduced hydrogen production. For instance, Fe at concentrations of more than 800mg L^{-1} respectively have been reported to decrease hydrogen production yields (Lee *et al*., 2001).

Substrate concentration

The response surface curve in Fig. 2A, 2D and 2E showed the interaction of substrate concentration and nutrients supplementation, initial pH and temperature on hydrogen yield respectively. Figure 2D showed that hydrogen yield increased from 0 mL/g TVS to 60 mL/g TVS with an increase in substrate concentration from 10g/L to 50g/L in interaction with the initial pH.

This result is in agreement with the findings of Lee *et al*. (2008) who observed an increase in both cumulative hydrogen production and hydrogen yield with increasing starch concentration. Increasing substrate concentration within an appropriate range can indeed enhance the ability of hydrogen producing bacteria to generate hydrogen by fermentation (Van Ginkel *et al*., 2001).

Initial pH

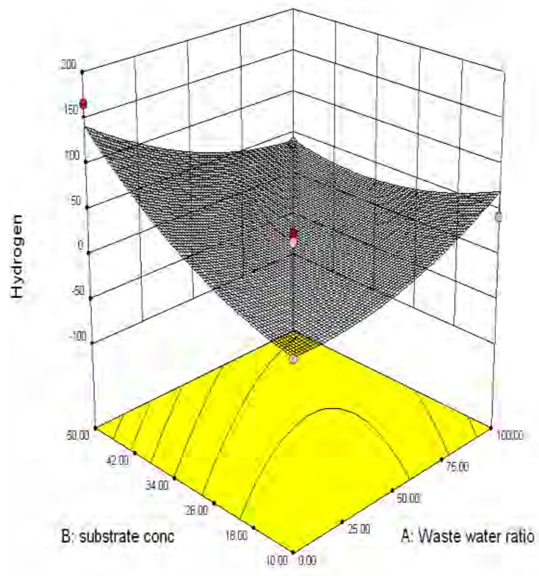
In this study, the interaction between initial pH and other process variables (nutrients supplementation, substrate concentration and temperature) showed a low significance on hydrogen yield (Fig 2B, 2D and 2F). Hydrogen yield increased slightly with an increase in pH from 5 to 6.5, and a maximum hydrogen yield of 40mL/g TVS was obtained at pH 6.8 and 10% nutrients supplementation.

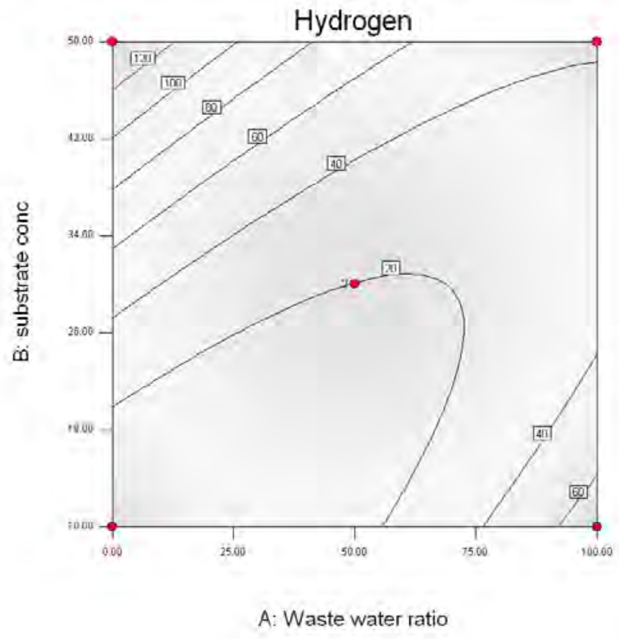
However, hydrogen yield decreased rapidly from 60 mL/g TVS to 20 mL/g TVS with further pH increase beyond 6.8. This may be due to interruption in hydrogen generation in which the high osmotic pressure forces the hydrogen producing bacteria to direct their metabolic activities away from hydrogen production to the stabilization of the internal environment. It has been reported that at a higher initial pH (above 7.0); a rapid hydrogen production will be accompanied by a rapid acid production which may deplete the buffering capacity and lowers the hydrogen production potential. On the other hand, at a lower initial pH (pH 4.5), the hydrogen producing microorganism will require a longer time to adjust to the conditions

which may also affect hydrogen yield (Khanal *et al*., 2004). Several studies have reported an optimum pH in the range of 5.5- 6.0 for fermentative hydrogen production (Zhang *et al*., 2003; Lee *et al*., 2008).

Temperature

Temperature is a vital operational parameter that affects hydrogen production as a result of its influence on physicochemical parameters such as pH as well as the growth rate and the survival of the hydrogen producing bacteria. At the same time it impacts the functionality of essential enzymes such as the hydrogenases (Sinha and Pandey, 2011). According to the RSM model, a maximum hydrogen yield of 200mL/g TVS was obtained at 30°C and 50g/L substrate concentration (Fig. 2E) while the hydrogen yield decreased rapidly with increasing temperature from 46°C to 55°C resulting to no detectable hydrogen yield .Given that the hydrogen producing bacteria present in the mixed culture are mostly mesophiles, such elevated temperatures will inhibit their growth and metabolic activity. It may also induce thermal hydrogenase denaturation which can hinder their ability to produce hydrogen (Sinha and Pandey, 2011).





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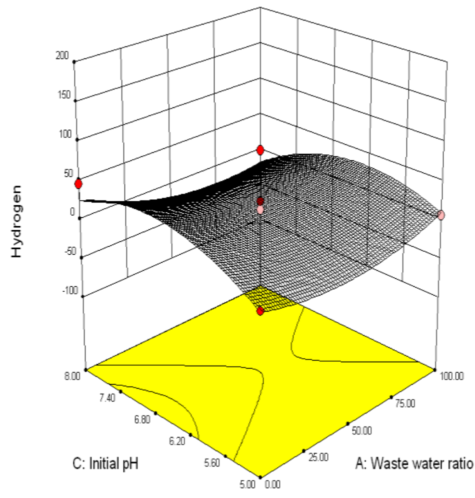

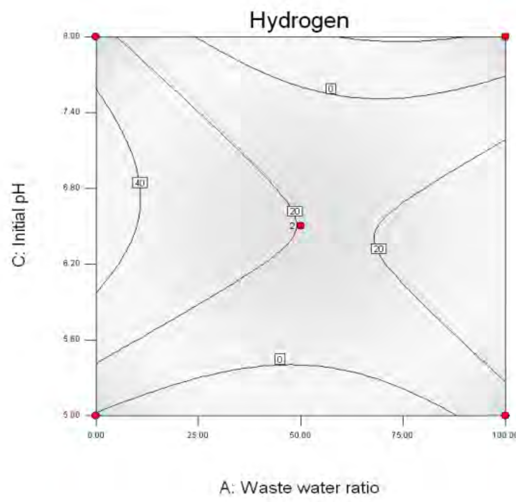

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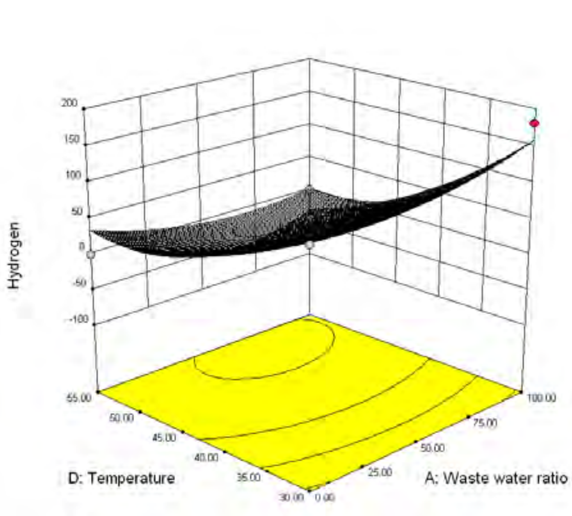

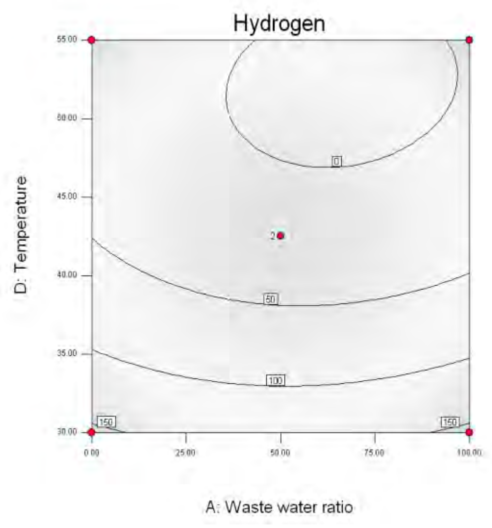

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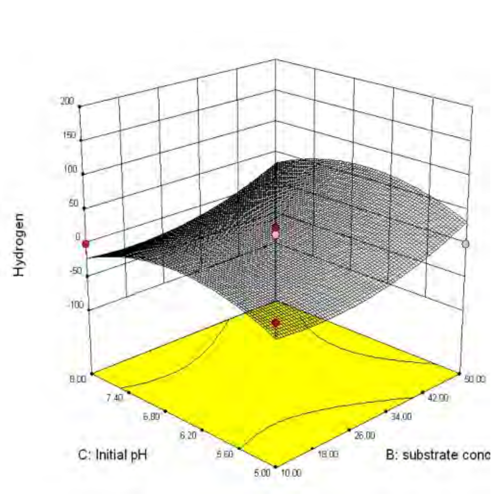

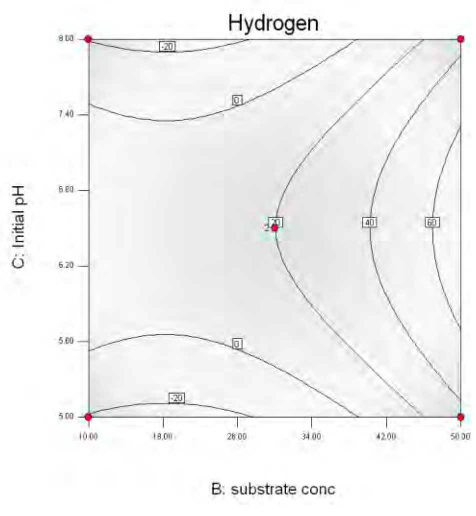

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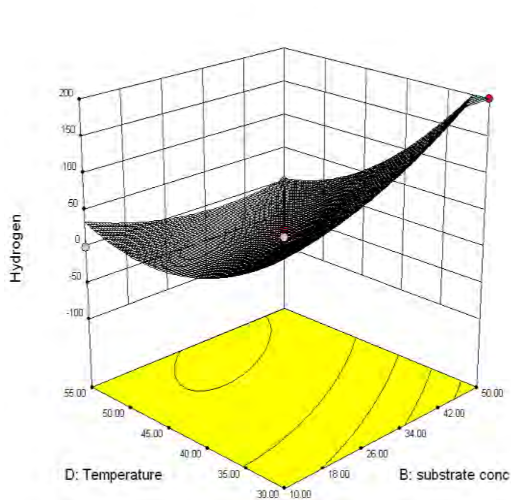

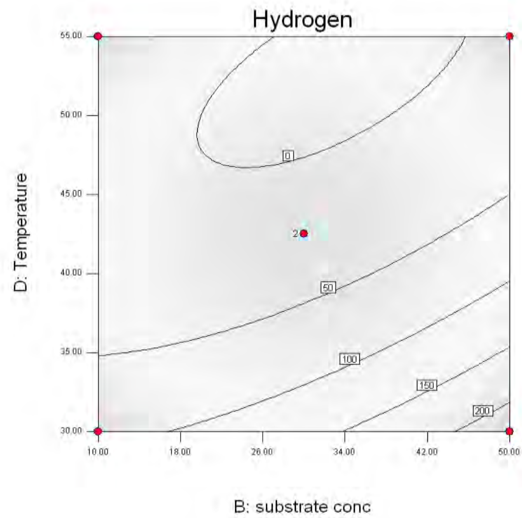

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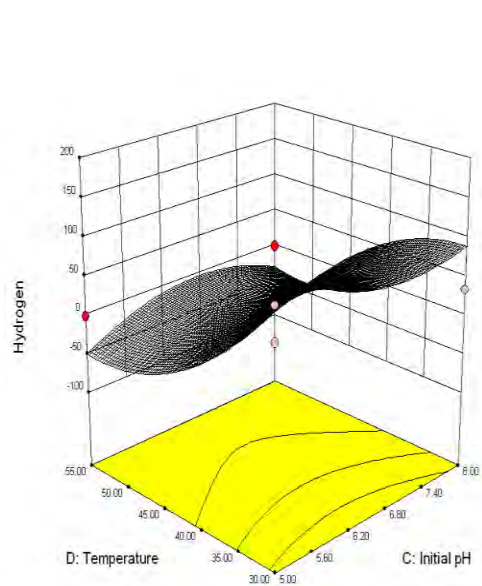

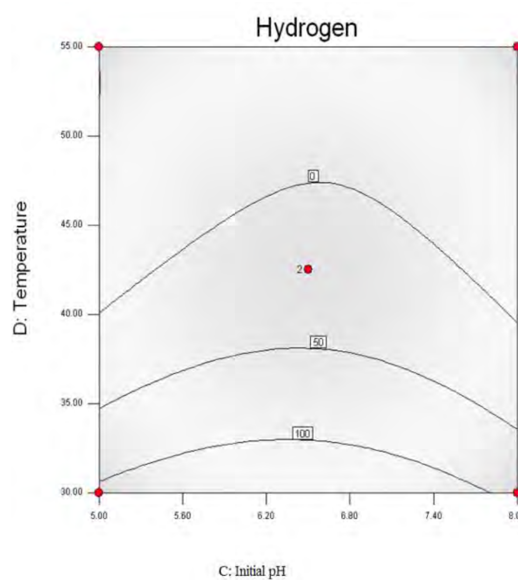

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Figure 2: Response surface curve and contour map plots showing the effects of nutrients supplementation, substrate concentration, initial pH and temperature on hydrogen yield.

3.3. Artificial neural network model

A committee made up of five ANNs was used. In adopting a committee machine, it was believed that this strategy could lead to significant improvement on the network prediction performance using little computation efforts. With this architecture, an Ensemble Averaging (EA) was adopted, where the individual model outputs for hydrogen yield are linearly combined rather than a Mixtures of Experts (ME), which involves the non- linear combination of the hydrogen output yields.

The result of the regression analysis of the committee model showed a coefficient of determination of 0.99 between the predicted and the experimental value. This demonstrated that the model was in good agreement with the experimental data and was able to capture the non-linearities in the process dynamics. Also, a coefficient of determination of 0.91 was obtained for the model validation. Figure 6 shows that there is a good fit between the experimental and the predicted hydrogen yield by the ANN committee.

An estimation of hydrogen production time profile using a single Neural Network was reported by Nasr *et al.* (2013). These authors used the input data of pH, initial substrate and biomass concentrations, temperature, process time and hydrogen yield from twenty five published studies and obtained a correlation coefficient of 0.98.

3.4. Comparison of the modelling abilities of RSM and ANN

ANN-GA and RSM techniques were comparatively assessed on their modelling accuracy and optimization efficiency. The plot of the predicted against the experimental data for the twenty six fermentation batches for ANN and RSM are presented in Figure 3A and 3B respectively.

As shown in Fig. 3A, it was observed that ANN plot had more data points closer to the diagonal line, thus illustrating its higher predictive accuracy over the RSM. On the other hand, results from Fig. 3B indicate that the RSM predictions had a greater deviation from the experimental value with more data points over estimating the hydrogen yield.

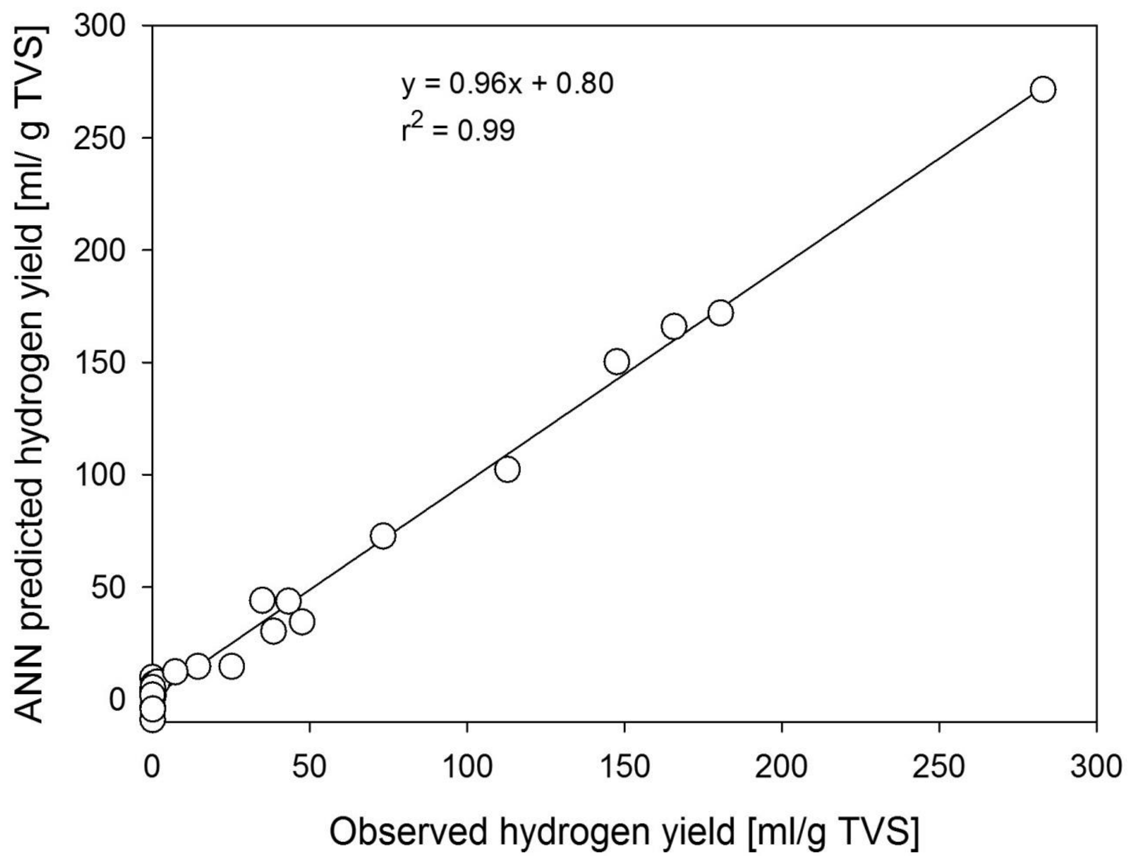


Figure 3A: Plot of ANN-GA predicted vs. experimental hydrogen yield for the twenty-six fermentation data sets.

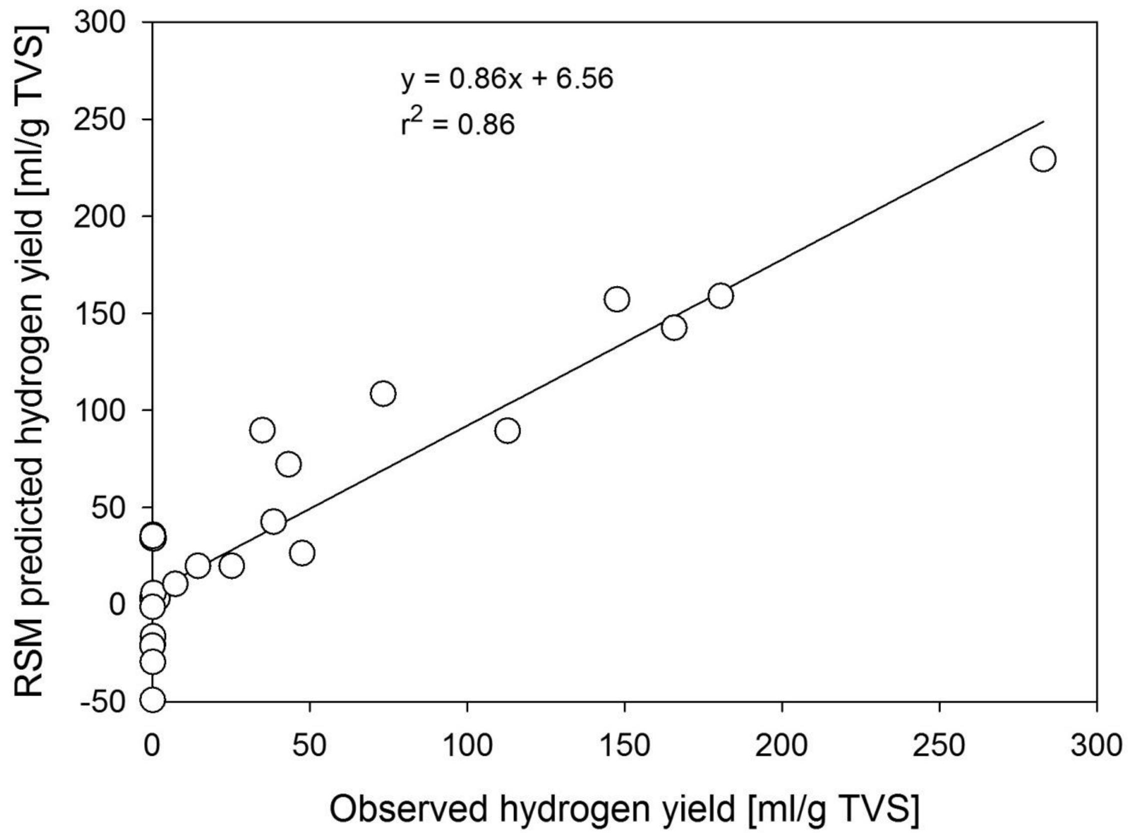


Figure 3B: Plot of RSM predicted vs. experimental hydrogen yield for the twenty-six fermentation data sets.

The coefficient of determination for the ANN committee and RSM was 0.99 and 0.85 respectively (Table 5) which further illustrates the ability of ANN to explain greater variability (99%) observed in the hydrogen fermentation process.

The optimized set points predicted by the ANN-GA are presented in Table 5. The optimal operational conditions predicted by both models were similar for substrate concentration and temperature but differed in nutrients supplementation and initial pH. These conditions were validated experimentally and a hydrogen yield of 186.72 mL/g TVS was observed against 201.01 mL/g TVS as predicted by the ANN-GA, with a yield of 159.32 mL/g TVS against 291.12 mL/g TVS predicted by the RSM (Table 5). This result showed that RSM grossly over estimated the hydrogen yield with a prediction error of 82.72% against 7.65% with ANN model prediction.

These findings further confirm the predictive accuracy of ANN to approximate the non linear interactions that exist in bioprocesses such as fermentative hydrogen production. Similar findings have been reported by Desai *et al*.(2008),Wang and Wan, (2009),Whiteman and Gueguim Kana, (2013).

Table 5: Comparison of the predictive ability of RSM and ANN models

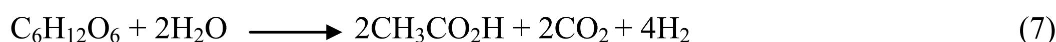
	Process parameters				Hydrogen yield (mL/g TVS)		
	Nutrients Supplementation (%)	Substrate concentration(g L^{-1})	pH	Temperature(°C)	Predicted value	Experimental value	% error
ANN	10	50	6.0	30	201.01	186.72	7.65
RSM	1.03	50	6.53	30	291.12	159.32	82.72

3.5. Pilot scale hydrogen production under optimized conditions

To understand the dynamics of the optimized hydrogen production process from potato peels at a large scale, a preliminary pilot process was investigated. Hydrogen production started at the 5th hour and increased rapidly to a peak concentration of 66.7% at the 11th hour of the fermentation (Figure 4A). The shorter lag phase observed could be attributed to the higher homogeneity and pH control achieved in the continuous stirred tank bioreactor.

Hydrogen concentration remained stable at 44 % from the 24th hour up to the 45th hour of fermentation, after which a rapid decline was observed at the 54th hour to 35%. The decline in production may be as a result of substrate depletion which in turn affected the growth and the activity of the hydrogen producing bacteria. A cumulative hydrogen production of 21.27L was observed at the 72 hour (Figure 4B) corresponding to a hydrogen yield of 239 mL/g TVS.

The metabolites produced were acetate (56.59%), butyrate (42.28%) and propionate (1.13%). The highest theoretical hydrogen yield of 4mol H_2 per mole of glucose can only be achieved when acetyl-CoA is metabolized to form acetate as the end product during fermentation (Hawkes *et al.*, 2002).



$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3CO_2H + 2CO_2 + 4H_2 \quad (7)$$

Our result indicates that the acetate type fermentation was favored in this process which may have contributed to the high hydrogen yield achieved during the pilot scale production. The low amounts of propionate also correlates with the high hydrogen yield obtained.

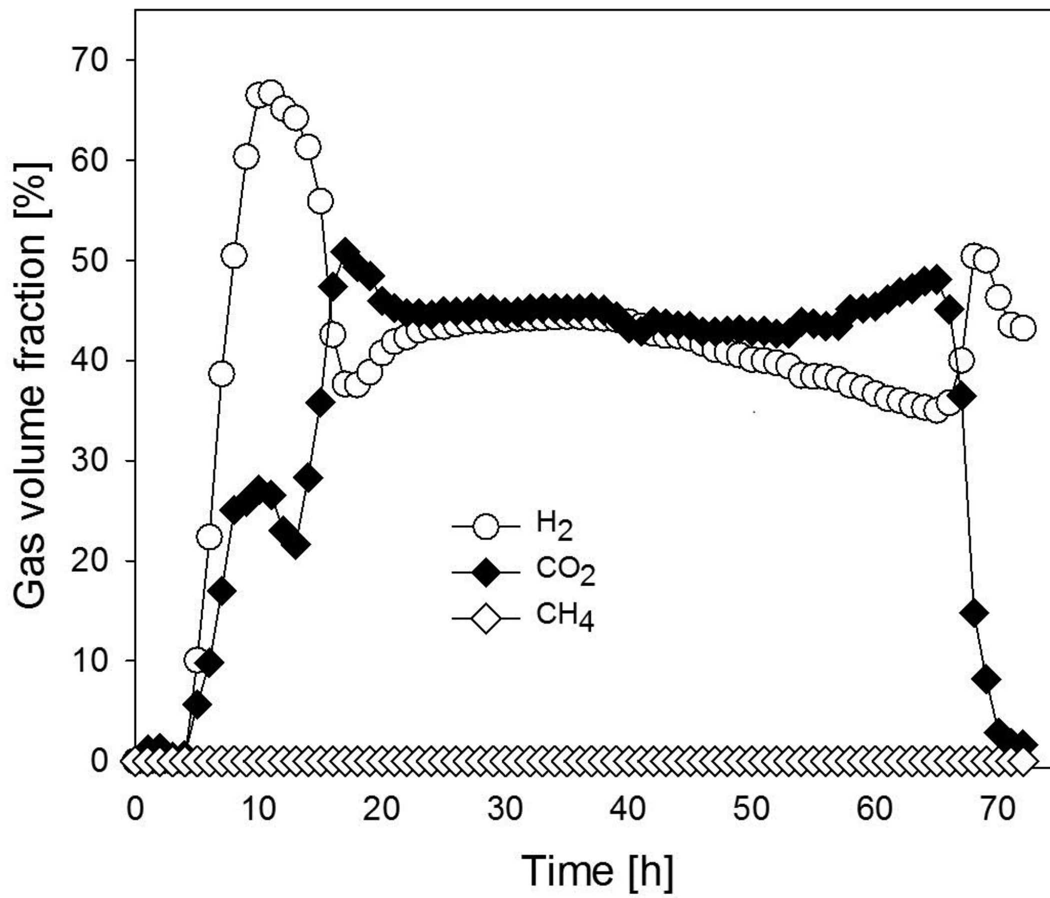


Figure 4A: Time course of biogas production during pilot scale production

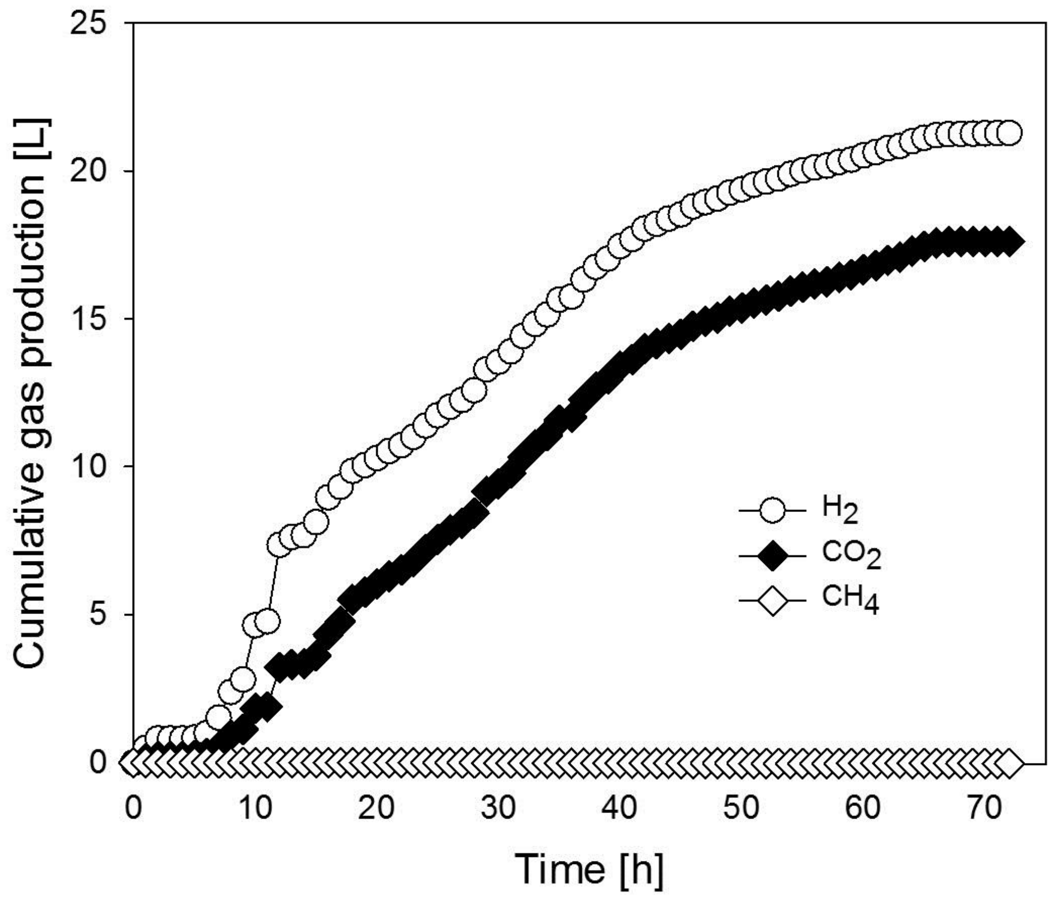


Figure 4B: Cumulative gas production at optimum condition over time

3.6. Conclusion

In this study, RSM and ANN were employed to model and optimize the operational conditions for hydrogen production using potato peels. Both models showed a satisfying coefficient of determination with 0.99 for ANN and 0.85 for RSM. However, the validation result showed that RSM grossly overestimated the hydrogen yield with a percentage error of 82.72% against 7.65% achieved with ANN. The maximum hydrogen yield of 239 mL/g TVS was achieved in a semi-pilot scale process. These findings highlights the relative superiority of ANN to model and optimize biohydrogen production and the prospect of scaling up biohydrogen production from potato peels.

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CHAPTER 5 - APPENDIX

Microbial community analysis of the semi-pilot hydrogen production system

1. INTRODUCTION

Pure and mixed microbial cultures are capable of breaking down substrates to produce hydrogen, however pure cultures application in fermentative biohydrogen production is limited because of the requirements for sterile conditions and stringent control of process conditions which may be difficult and expensive especially at industrial scale (Kapdan and Kargi, 2006; Ren *et al*., 2006; Argun and Kargi, 2009).

On the other hand, mixed culture could result to a better process production performance due to the possibility of utilizing complex, non sterile and cheap waste as substrates (Luo *et al*., 2010; Guo *et al*., 2010). In addition, the use of mixed culture may contributes to an improvement in hydrogen yield and production rate because of the presence of different microbial species which are likely to possess better hydrolytic properties and more robust to adapt to changes in process conditions (Hawkes *et al.,* 2002; Hallenbeck and Ghosh, 2009). Other benefits includes the possibility of co fermentation of different substrates, high microbial diversity, high tolerance to the feedstock indigenous microbes, process stability resulting into better substrate conversion efficiency and hydrogen yield(Kotsopoulous *et al*., 2006; Kongjan *et al*., 2010; Cheng *et al*., 2011).

Mixed culture inoculum can be obtained from natural sources such as animal dung, soil and sewage sludge and can also originate from the feedstock used (Li and Fang, 2007). The proliferation of non hydrogen producing bacteria and hydrogen consuming bacteria in the mixed culture often times results into a complete shift in the metabolic activities during fermentation which may have a detrimental impact on the hydrogen yield (Ren *et al*., 2007a,

b). Also the presence of non hydrogen producing bacteria can affect the overall performance of the fermentation process as the bacteria could compete for the substrates thereby reducing hydrogen yields (Jo *et al*., 2007). These challenges necessitates the need to eliminate the hydrogen consuming bacteria and non hydrogen producing bacteria in the mixed culture to achieve improvement in hydrogen production through the process of inoculum pre-treatment(Hung *et al*., 2011).

Numerous variations exists regarding the hydrogen yield and production rate in dark fermentation from literature which may be as result of the difference in the process conditions, substrate type, inoculum source and more importantly the microbial community structure(Kongjan *et al*., 2010). The microbial community structure is influenced by the source of inoculum, operating conditions, substrate type and concentration; therefore the knowledge of the microbial community composition during fermentation will be an essential key to finding the optimal process conditions towards enhancing hydrogen yields (Hung *et al*., 2011). Reports from literature indicated that hydrogen yield and production rate are directly related with the microbial community structure in essence the amount of the dominant hydrogen producing bacteria in the bioreactor. Therefore understanding the effects of the operational parameters as well as the microbial community diversity will give valuable information on the optimization of hydrogen production for the development of a viable commercial hydrogen production system (Cheng *et al*., 2011).

Traditional methods of microbial identification include isolation of pure cultures and investigating the morphological, biochemical, and physiological characteristics of the isolates. However such method may be cumbersome and not completely reliable as some microbes may be difficult to culture on growth media (Amann *et al*., 2005). Recently, advanced molecular techniques have been used to study the microbial community structure in fermentative hydrogen production under various process conditions such as temperature,

HRT, pH, and substrate type (Hung *et al*., 2008; Rittmann *et al*., 2008). Such techniques includes Polymerase chain reaction- Denaturing gradient gel electrophoresis (PCR-DGGE) which is commonly used to examine the dynamic changes in the mixed culture during fermentation (Jo *et al*., 2007; O-Thong *et al*., 2009; Liu *et al*., 2009; Lin *et al*., 2011) Terminal restriction fragment length polymorphism(Castello *et al*., 2009; Ueno *et al*., 2006), Fluorescence in-situ hybridization (FISH) (Leano *et al*., 2012), PCR-Clone library (Chaganti et al., 2012; Rafrafi *et al*., 2013; Tomazetto and Oliviera, 2013; Nitipan *et al*., 2014), Pyrosequencing of marker genes (Im *et al*., 2012; Lu *et al*., 2012) Quantitative real time polymerase chain reaction(qPCR) (Chang *et al*., 2008; Tolvanen *et al*., 2008; Cheng *et al*., 2011; Leano *et al*., 2012) and more recently next generation sequencing (Boboescu *et al*., 2014). These methods are used to evaluate the structure and the phylogenetic affliation of the microbial community members.

The 16SrDNA region is very conserved and stable in prokaryotes which permit its use as a universal molecular marker for the identification of microbial community structure in environmental sample (Muyzer *et al*., 1993). Molecular techniques that targets the 16srRNA gene coupled with culture based techniques can offer valuable information on the diversity and complexity of the microbial community structure (Krakat *et al*., 2010). Clone libraries are increasingly been used to analyze microbial population diversity with the benefits of a more quantitative profile of the microbial community (Kiely *et al*., 2010; Krakat *et al*., 2011; Qiu *et al*., 2011).

Despite many reports on the optimization of process conditions in fermentative biohydrogen production (Mohan *et al*., 2009; Guo *et al*., 2011; Han *et al*., 2012; Won *et al*., 2013), few studies have focussed on the analysis of the microbial composition during fermentation, this is very important in order to identify and understand their roles during fermentation (Rafrafi *et al*., 2014).

Therefore, this study investigates the microbial community structure of a pre-treated anaerobic sludge inoculum for hydrogen production from potato peel waste. A snapshot of the bacteria population present during the peak production phase of the semi-pilot scale batch fermentation was analyzed via the construction of a 16SrRNA clone library.

2. Materials and Methods

2.1 Sample collection

Aliquot of the fermentation broth was taken during the peak production phase of the semi-pilot production (as described in Section 2.9 of Chapter 5). The sample was stored at -20°C for further analysis.

2.2 DNA Extraction and PCR Amplification

DNA was extracted from 5mL aliquots of the fermentation broth sample using a Power soil DNA Kit (MO Bio Laboratory, Inc., USA) according to nu c u 's ns uc ns Amplification of the 16S rRNA gene was carried out using the following universal primer pair: 27F, (5'-AGAGTTTGATCMTGGCTCAG-3'), and 1492R, (5'TACGGYTACCTTGTTGTTACGACTT-3') (Lane, 1991). PCR was performed using KAPA 2G fast DNA polymerase (KAPA Biosystems, South Africa) according to the nu c u s' ns uc ns n 20 µL c n c n n n 5 µL DNA with a G-STORM thermal cycler (Vacutec, South Africa). PCR protocols includes an initial denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 15 s, annealing at 53 °C for 25 s and elongation at 72 °C for 25 s; with a final extension step of 72 °C for 5 min. Amplicons were resolved on 2% agarose gel stained with SYBR Safe (Invitrogen, Canada) under UV lights for 45 min and the size of the amplicons were verified using a 1Kb DNA ladder (Thermo Scientific, USA). PCR products of the expected size were excised and purified using the MinElute Gel Extraction kit (Qiagen, Netherlands).

2.3. Clone library construction

The purified product was ligated into the pCR 2.1 vector using a TA Cloning kit (Invitrogen, Canada) as specified by the manufacturer, following transformation into competent *E. coli* (TOP10) cells. A total of 100 positive transformants (single white colonies) were randomly selected from Luria Bertani (LB) plates containing 50 µg/mL Ampicillin and 40 mg/mL X-Gal and were inoculated into 5 mL of LB broth containing 50 µg/mL Ampicillin. Samples were incubated for 16 h at 37 °C in a shaking incubator (>225 rpm). The Plasmid DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen, Netherlands) as specified by the nu c u Ins s d d y PCR us n p s d sp c c p s M13F (5’-GTAAAAGGACGGCCAG-3’) nd M13R (5’-CAGGAAACAGCTATGAC-3’) nd PCR conditions for amplification were 2 min denaturation at 95 °C **;** followed by 30 cycles of 95°C for 15 s, annealing at 55 °C for 20 s, elongation at 72 °C for 20s; and a final extension step at 72 °C for 5 min followed by 2% agarose gel electrophoresis as stated above.

Only the positive clones with the expected inserts were selected and subjected to further screening with the amplified rDNA restriction analysis (ARDRA) to select clones for sequence analysis. Plasmids containing inserts with the expected size were subjected to restriction analysis using BfoI and HindIII restriction enzymes (Thermo Scientific, USA) n nu c u s’ u d n s D s d DNA n s s d n 2 % agarose gel stained with SYBR safe (Invitrogen, Canada). A 1 Kb DNA ladder (Thermo Scientific, USA) was used to analyze the band pattern.

2.4 Sequencing and Phylogenetic analysis

Clones representing distinct restriction patterns identified by the image analysis were selected and sequenced at Inqaba Biotech (Pretoria, South Africa) in the forward and reverse directions using the M13 primers. A 97 % sequence match was the minimum criteria used for the assignment of an operational taxonomic unit (OTU) following BLASTn analysis using

the nucleotide collection of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

The sequences were aligned using Cluster W and a phlogenetic tree was constructed using the neighbour joining method with bootstraps test for 1000 replicates in Mega 6.0 software (Tamura *et al*., 2013). Rarefaction curve was constructed by plotting the number of clones representing the distinct phylotypes (with greater than equal to 97% threshold) to determine whether the sequenced clones adequately represent the community diversity (Hughes *et al*., 2001).

3. Results and Discussion

3.1. Microbial community structure during hydrogen production

The microbial community structure during hydrogen production using potato peels waste was investigated using the PCR-clone library. A total of 55 positive clones were isolated and are grouped into ten operational taxonomic units (OTUs) as defined by the ARDRA analysis (Table 1). The analysis of the ten OTUs established showed that 85% of the clones had a sequence similarity that is greater than or equal to 97% with only 15% showing sequence similarity lesser than 97%. These similarity nd x s ≥ 97% and ≤ 94% are reliable to match clones at species and genus level respectively (Santis *et al*., 2007). Although some authors have argued on the limitation of PCR based method which may arise as a result of inefficient DNA extraction, variation in the copy of numbers of bacteria 16SrRNA gene, sensitivity to the DNA template concentration, primers sensitivity and amplification bias (Nocker *et al*., 2007), PCR based methods offers the merits of a high phylogenetic resolution as it helps to determine the closest phylogenetic neighbour within the community (Singleton *et al*., 2001).

ARDRA is a useful method of reducing the time and cost of sequencing for microbial diversity analysis (Ramos *et al*., 2010), however oftentimes analysis of complex microbial

community may show restriction patterns which may be difficult to resolve and differentiate on agarose gels (Dunbar *et al*., 1999). The detection limit of ARDRA technique was reported to be approximately 10^5 cells /ml which are the same as the detection limit of epifluorescence microscopy, confirming the accuracy and reliability of the technique (Krakat *et al*., 2010). Also, Nitipan *et al*. (2014) obtained similar result using the PCR-clone library and Fluorescence in situ hybridization (FISH) for microbial community structure analysis.

The OTUs observed in this study can be divided into two classes of *Clostridia* and *Bacilli*. Eighty six percent of these were related to the genus *Clostridium* and matched six species listed in the ascending order of predominance: *C. aminovalericum*, *C. intestinale* , *C. tertium*, *C. sartagofome* , *C. beijerinckii* and *C. butyricum*, the remaining 2% closely matched with *Anaerobacter mobilis* as confirmed by the phylogenetic tree (Figure 2). Also, six percent of the clones were found to be ninety nine percent affiliated with *Bacillus thermoamylovorans* as shown in Figure 1.

Among the six OTUs of the genus *Clostridium*, 44% of the clones closely matched with *C. buyricum* which indicates the dominance of this species in the hydrogen production microbial community. The community structure and the diversity of this production system were similar to earlier reports from other authors which confirmed the genus *Clostridium* as the dominant hydrogen producing bacteria (Prasertsan *et al*., 2009; Matinguer *et al*., 2008; Moreno-Davilla *et al*., 2010; Lin *et al*., 2010; Mohan *et al*., 2011). Goud *et al*. (2012) reported the predominance of *Clostridia* and *Bacilli* in the biohydrogen production microbial community under different process conditions for 1435 days. Several species of *Clostridium* are known for their fermentative hydrogen production capabilities including *C. acetobutylicum* (Goud *et al*., 2012), *C. butyricum* (Noparat *et al*., 2012), *C. pasterianum* (Brosseau *et al*., 2007).

Operation taxonomic distribution(OTUs) distribution of the clones generated from the fermented broth.

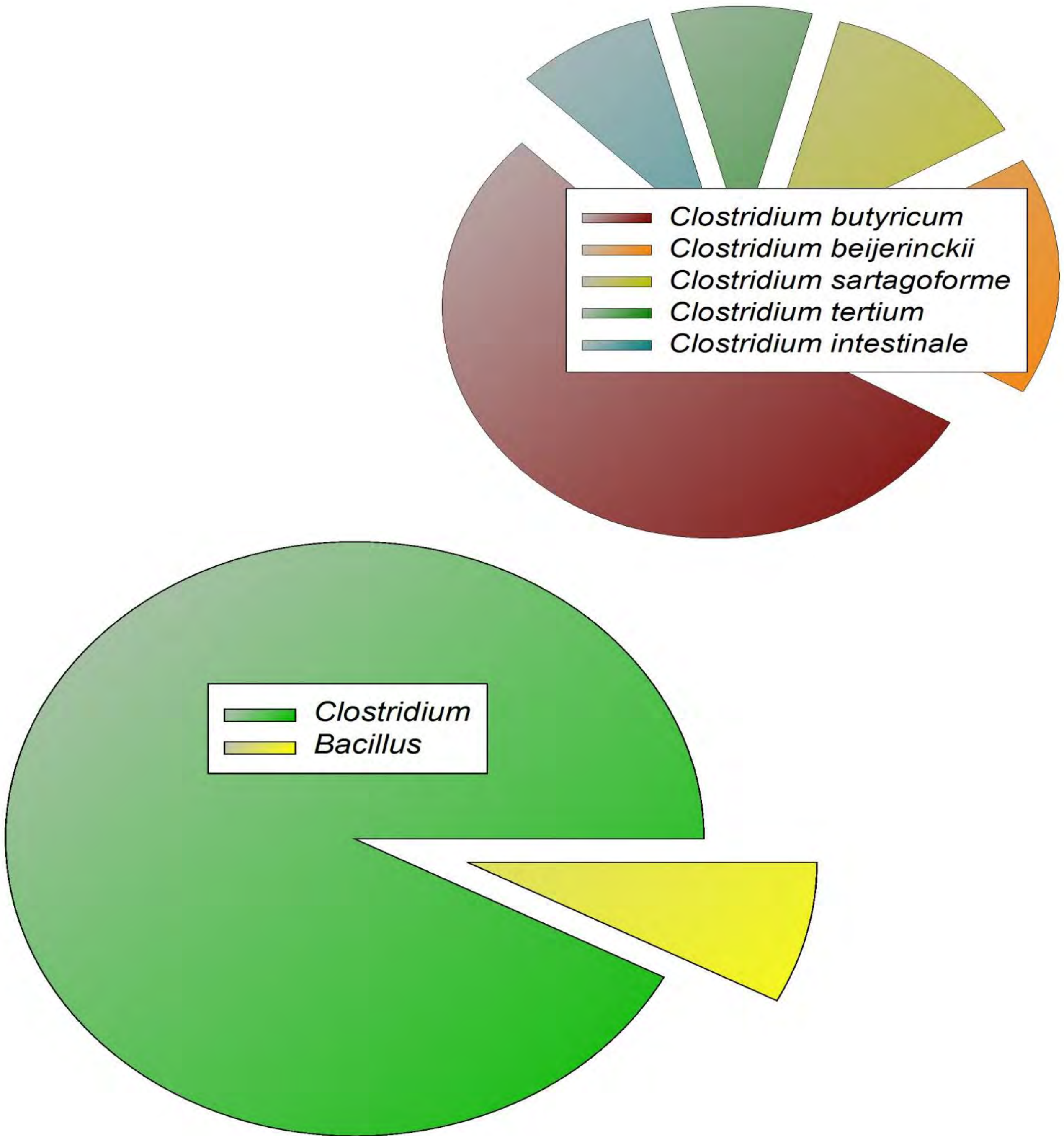

Figure 1: 16SrRNA community profile of the hydrogen production from potato peel waste. Operation taxonomic distribution(OTUs) distribution of the clones generated from the fermented broth.

Table 1: Phylogenetic sequence affliation to the closest relative of the amplified 16SrRNA sequence representing clones established from the hydrogen production reactor.

Groups	No of clones	Size (bp)	Closest Related Match	Sequence similarity (%)	Accession Number
OTU -1	6	1568	*Clostridium sartagofome* JCM 1413	99	AB971796.1
OTU- 2	1	1573	*Anaerosporobacter mobilis*	92	AY534872.2
OTU- 3	8	1570	*Clostridium beijerinckii* ATCC 35702	98	CP006777.1
OTU- 4	24	1569	*Clostridium butyricum* NCIMB 8082	99	X68178.1
OTU- 5	1	1582	*Clostridium aminovalericum*	93	KC331198.1
OTU- 6	4	1474	*Clostridium tertium* JCM 6289	99	NR113325.1
OTU- 7	3	1546	*Bacillus thermoamylovorans* N12-2	99	HM030742.1
OTU- 8	1	1160	Uncultured bacteria	98	EU775625.1
OTU- 9	2	1464	Uncultured bacteria	94	GQ133782.1
OTU- 10	4	1541	*Clostridium intestinale*	94	AY781385.1

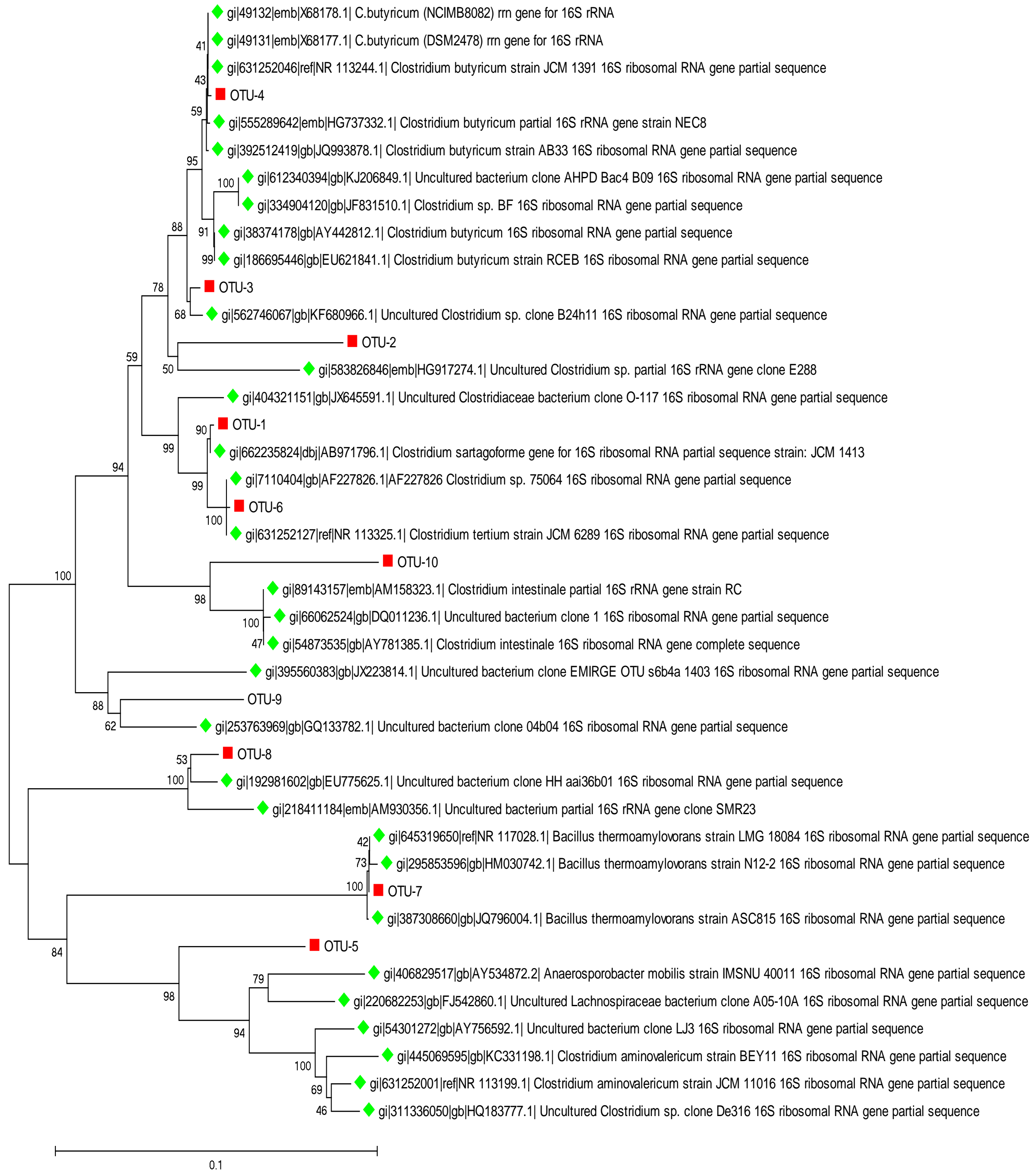

Figure 2: Unrooted phylogenetic tree showing the affiliation of 10 OTU's identified in the hydrogen bioreactor clone library based on 16S rRNA gene sequences of representative clones in comparison to most closely related sequences deposited in GenBank. Numbers shown at nodes indicate calculated bootstrap values (only values >40% are shown). The scale bar indicates 10 estimated changes per 100 nucleotides.

Members of the genus Clostridium are gram positive, motile, obligate anaerobic endospore forming rods. *Clostridium* species are ubiquitous, versatile in terms of their metabolic capabilities and can be isolated from various environments such as soil, compost, faeces, sludge, intestinal tracts of human and animals (Collins *et al*., 1994; Rainey *et al*., 2009). They are capable of metabolizing carbohydrate to produce mixtures of organic acids and alcohols with optimum growth at pH 6.5-7 and 30°C -37°C. Species such as *C. butyricum*, C. *beijerinckii*, and *C. sartagofome* are capable of producing hydrogen and large amounts of acetic, butyric and formic acid during fermentation (Weigel *et al*., 2005). The growth of *C. butyricum* is stimulated by carbohydrate fermentation and plays a major role in the hydrolysis of starch (Chesson and Forsberg, 1988). *Anaerosporobacter mobilis* are gram positive strictly anaerobic endospore forming rods which ferments glucose and the major end products are acetate, formate and hydrogen (Collins *et al*., 1994). Although most studies have focussed on the dominant hydrogen producing bacteria such as *Clostridium* spp, however the presence of other associated bacteria such as *Bacillus, Streptococcus, Escherichia coli, Klebsiella* spp have been reported (Hung *et al*., 2011).

In this study, 6% of the isolated clones were affiliated with *Bacillus thermoamylovorans*, *Bacillus* species are straight or slightly curved gram positive, facultative anaerobic endospore forming rods (Logan and Berckeley, 1981). The endospores are very resistant to many agents such as heat, radiation, nutrients depletion, chemicals and can survive for a long duration of time (Atrih and Foster, 2001). *Bacillus* species have been reported to play some roles in hydrogen production by contributing to the hydrolysis of the substrate resulting into a higher substrate conversion efficiency and improvement in hydrogen yield (Ueno *et al*., 2006). Hung *et al*. (2010) also reported the role of *Bacillus* species in depleting the amount of dissolved oxygen during the lag phase of hydrogen production to create an optimum growth environment for the strictly anaerobic *Clostridium* species.

The rarefaction curve (Figure 3) showed that the clones analysed showed a narrower taxonomic diversity, this may be as a result of the inoculum pre-treatment which enriched for only the endospore forming hydrogen producing bacteria as a result of their resistance to the heat treatment. Hydrogen consuming methanogens were not detected in this study which further confirms the efficiency of the hybrid pre-treatment technique.

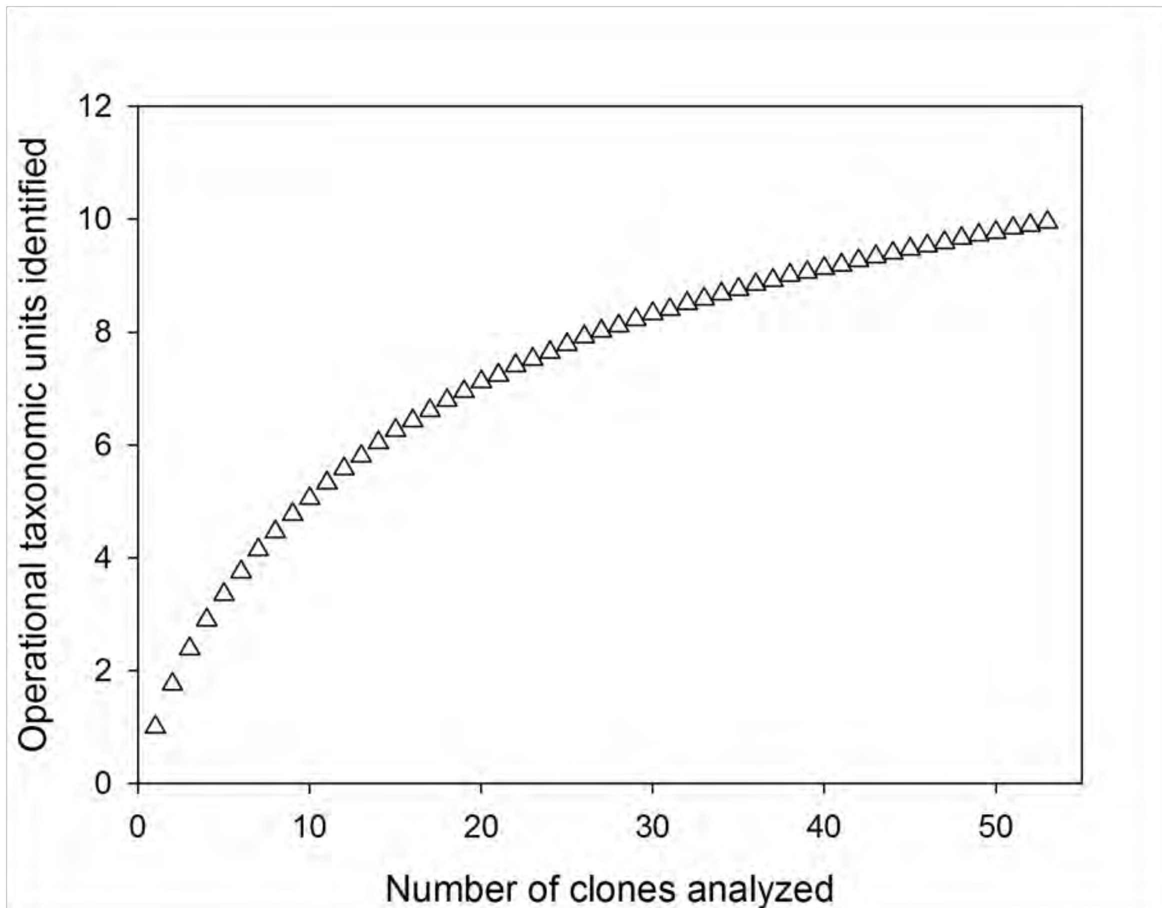

Figure 3: Rarefaction curve of the fermentor sample showing the relationship between the number of OTUs and the number of clones collected.

4.0 Conclusion

The microbial community of a semi-pilot scale hydrogenproduction from potato peel wastes was investigated using the PCR-Clone library. The study indicated the dominance of the genus *Clostridium* in the hydrogen production process and the phylogenetic distribution consists of species such as *C. butyricum* (44%), *C. beijerinckii* (15%), *C. sartagofome* (11%), *C. tertium* (15%), *C. intestinale* (7%), *C. aminovalericum* (2%) and only 6% were affliated with *Bacillus thermoamylovorans*. It appears that the hybrid inoculum treatment was effective in enriching for only the endospore forming hydrogen producing bacteria and eliminated the hydrogen consuming methanogens as none was detected. This study may help in the design of functional mixed inoculum using specific substrates such as potato peels waste for hydrogen production.

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CHAPTER 6
Concluding Remarks

6.1. Summary and Conclusions

The realization of the potential of biohydrogen production technology will contribute to the development of global economy by facilitating sustainable energy supply alongside the reduction of greenhouse gases emission and environmental pollution. The ultimate goal of the fermentative hydrogen production system is to achieve higher yields for it to be considered as an economically viable renewable energy system. This research focussed on different approach towards achieving this goal and the major highlights include:

- The development of a suitable mixed inoculum for biohydrogen production: A hybrid inoculum pre-treatment of pH and Autoclave was developed via modelling and optimization using the Response Surface methodology. The model suggested that mixed culture treatment at a pH value of 8.9 followed by autoclaving (121°C) for 15 minutes can enrich for the endospore forming hydrogen producing bacteria while eliminating the activity of the hydrogen consuming methanogens. This inoculum development technique will be one of the key strategies to enhance hydrogen production by accelerating substrate hydrolysis and improving the process stability. This strategy will positively impact the start-up and the overall efficiency of the hydrogen production process. Preliminary scale up using this hybrid inoculum pretreatment indicated biogas production with hydrogen fraction of approximately 53% which further confirms the reliability of this method. The hybrid pH and Autoclave pretreatment is a promising technique for industrial application with the advantage of short time requiring less energy input and cost to enrich hydrogen producing microorganism in the mixed inoculum.

- The effect of the combined pH and microwave treatment on the hydrogen production mixed inoculum was modelled and optimized using the RSM. Microwave treatment of the sludge at pH 11 for 2 min at 860W resulted in the suppression of the activity of the hydrogen consuming methanogens and a 32.4% increase in hydrogen yield. Methane production was suppressed indicating the effectiveness of this combined pre-treatment method to enrich hydrogen producing bacteria. Furthermore, this technique could be adapted for industrial production due to its low cost and energy saving approach as evident from the short microwave treatment duration compared to conventional heating which has been widely used.
- Semi pilot scale assessment of this technique indicated a maximum hydrogen molar yield of 1.78mol H_2/mol glucose in the absence of pH control compared with 2.07 mol H_2/mol glucose in the pH controlled process. These results demonstrate the influence of pH control for enhanced hydrogen production.
- The feasibility of biohydrogen production from potato peels waste was demonstrated. A semi pilot process with the pre-treated inoculum under the optimized condition of 50g/l and 10% nutrient supplementation at 30°C, pH 6 gave a maximum hydrogen yield of 239.94 mL g^{-1}TVS. This hydrogen production technology using potato peel wastes could be an attractive system to realize the goal of industrial scale production with the merits of low process cost due to the abundance of the feedstock and its consideration as wastes with associated disposal problems. South Africa and other countries with high agricultural production capabilities can effectively improve their economy through the incorporation of biohydrogen production into agro industry. Hydrogen production from potato peel waste will also help to reduce environmental pollution by its use for energy generation.

- The commercialization of any bioprocess relies on its capability for industrial scale-up. The assessment of the semi-pilot scale up of hydrogen production using potato peel wastes in this study provides an analytical frame work to understand the dynamics and the challenges of this process towards achieving the commercialization of this technology.
- Process modelling and optimization tools including Response Surface methodology and the Artificial Neural Network employed in this research provides a more reliable approach to develop biohydrogen production and highllited the efficiency of using Artificial Intelligence based strategies for bioprocess modelling and optimization.
- A more functional microbial consortium can be developed based on the result from the microbial community anlysis in this study specifically for hydrogen production from potato peel wastes with improvement in process stability and hydrogen yield.

6.2. Future research and suggestions

Significant results was achieved in this study as regards the optimization of production yield and the development of a stable requisite inoculum which is a significant step on the path to industrial scale up of this renewable energy system. Despite many reports on fermentative biohydrogen p duc n s 'n y uu ', s n s u d s address in order to achieve this goal. Therefore more research should focus on:

- ❖ Improvement in the capability of the hydrogen producing bacteria through techniques such as Metabolic Engineering and immobilization especially for continuous production.
- ❖ Integration of hydrogen production with other renewable energy generation systems such as biogas, microbial fuel cells (MFC), and microbial electrolysis cell (MEC) could help to maximise substrate conversion efficiency and energy recovery from this feedstock following the analysis of the process economics.

- The design and development of novel bioreactors that can address the problem of biomass wash out, process instability and encourage hydrogen generation within a shorter hydraulic retention time requires more research for practical application of biohydrogen at a large scale.
- More research should focus on other areas such as hydrogen energy storage, hydrogen transportation and power generation in order to achieve this goal.

Thesis supplementary Figure

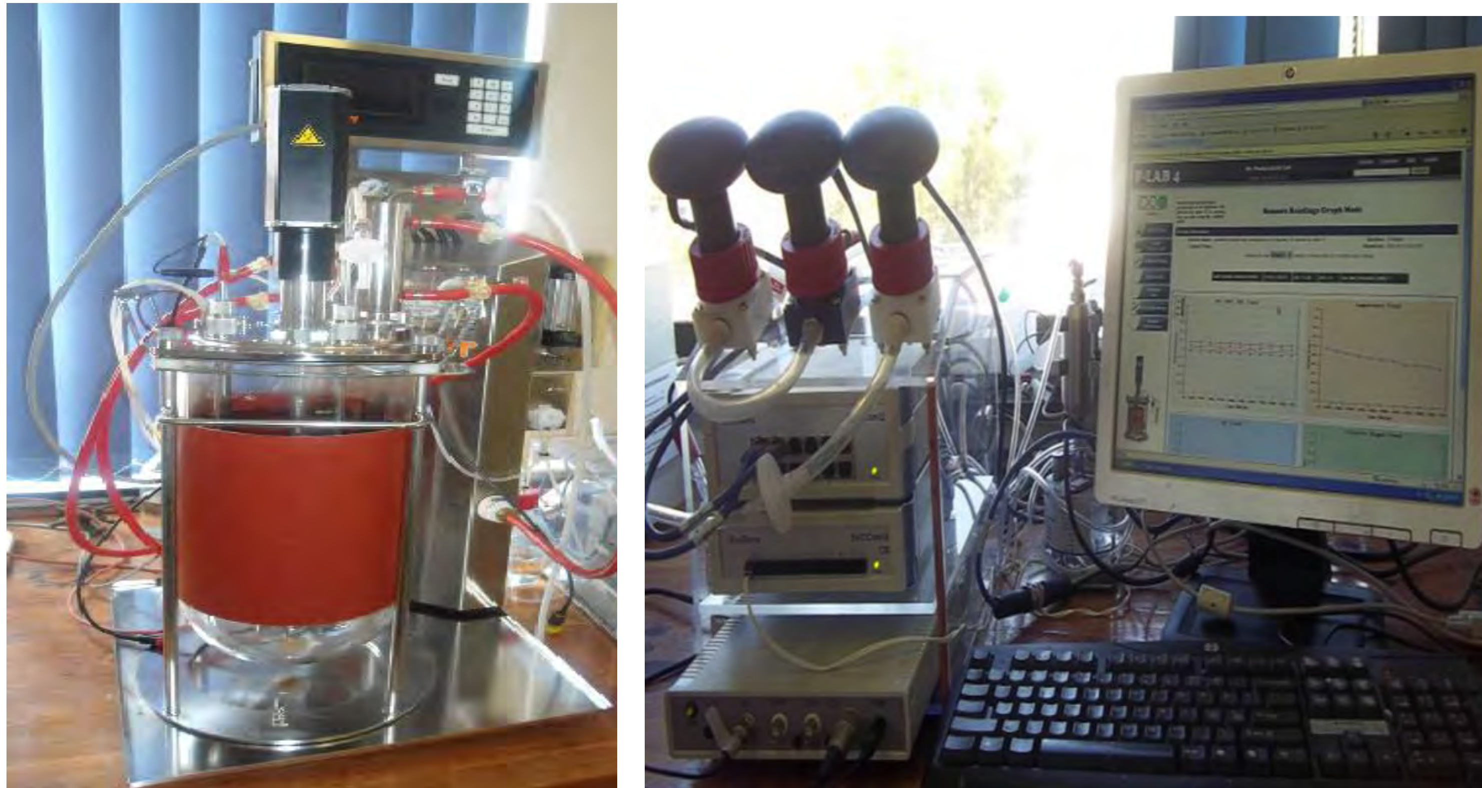

Figure 1: 10L CSTR bioreactor (Labfors Infors) for the semi pilot scale hydrogen production