Bioprocess development for hydrogen production by dark fermentation using waste sugarcane leaves

by

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Preface

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

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

Signed:

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

Date: 09 December 2015

Declaration 1: Plagiarism

I, Preshanthan Moodley, declare that:

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

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

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

(vi) this dissertation is primarily a collection of material, prepared by myself, submitted for publication or presented at conferences. In some cases, additional material has been included;

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Declaration 2- Publications

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

1. Assessment of three optimized models for the production of xylose and glucose from waste sugarcane leaves using different acid-based hybrid pretreatments for biohydrogen production. Submitted to *Biochemical Engineering Journal*. Under review. (Chapter 3).

2. Optimization of Physico-Chemical Parameters for Biohydrogen Production from Waste Sugarcane Leaves via Dark Fermentation- A semi pilot scale assessment. Submitted to *Biomass and Bioenerg*. Under review. (Chapter 4).

3. Techno-economic Analysis of a Large Scale Production of Biohydrogen from Waste Sugarcane Leaves using Dark Fermentation. Submitted to *Biofuel Research Journal*. Under review. (Chapter 5).

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Conference proceedings and contributions

1. Moodley P, Kana EBG (2015) Comparative study of dilute acid pretreatment techniques for fermentable sugar production from sugarcane leaves (*Saccharum officinarum*). Journal of Fundamentals of Renewable Energy and Applications 5(6): 61. International Conference on Green Energy and Expo, Orlando, USA, 21-23 September 2015, Oral presentation; http://dx.doi.org/10.4172/2090-4541.C1.005.

2. Moodley P, Kana EBG (2015) Assessment of three optimized models for the production of xylose and glucose from sugarcane leaves using acid –based hybrid pretreatments for biohydrogen production. Global Cleaner Production and Sustainable Consumption Conference. Journal of Cleaner Production, Elsevier. Barcelona, Spain, 1-4 November 2015. Poster presentation.

3. Moodley P, Kana EBG (2016) Bioprocess development for hydrogen production by dark fermentation using waste sugarcane leaves. South African Society for Microbiology (SASM) Biennial Congress. Durban, South Africa, 17-20 January 2016, Oral presentation.

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Abstract

One of humanity's greatest challenges in future decades will be the need to develop a renewable, sustainable, and environmentally friendly energy source to replace our high dependency on fossil fuels, which are rapidly depleting. The generation of hydrogen from biological sources (biohydrogen) has emerged as a promising alternative to fossil fuels due to its high energy content and clean combustion profile. Biohydrogen can be generated via dark fermentation using lignocellulosic biomass, such as sugarcane leaves, as a feedstock. Approximately 640 million tons of waste sugarcane leaves are generated annually and burned prior to harvest, posing serious health concerns. The production of xylose and glucose from waste sugarcane leaves via acid-based pretreatments were optimized and are reported in this study. In addition, the production of biohydrogen from these substrates were modeled and optimized, and a techno-economic analysis of a large-scale biohydrogen plant was performed.

Six models were optimized for the production of xylose and glucose using HCl, H_2SO_4, and HNO_3. The input variables for each model consisted of acid concentration, temperature, solid to liquid ratio (S/L), and heating time. All models showed coefficients of determination (R^2) above 0.78, indicating that they were adequate for navigating the optimization space. Optimization of the process gave xylose and glucose yields of 78 g/L and 11.48 g/L for HCl-, 50.75 g/L and 7.15 g/L for H_2SO_4-, and 30.82 g/L and 3.99 g/L for HNO_3-based hybrid pretreatments. The HCl-based pretreatment, using the optimized conditions of 4.90% HCl at 99 °C for 84 min, with an S:L ratio of 47.26%, showed high solubilization of hemicellulose and had the shortest heating time. The interactive effect of these input parameters on the sugar recovery pattern revealed that increasing the acid concentration from 0.5 to 5.0% and the heating temperature from 60 °C to 100 °C resulted in higher yields of glucose and xylose.

Biohydrogen production from the pretreated waste sugarcane leaves was then modeled and optimized using a Box–Behnken design. Substrate concentration (8–24 g/L), inoculum concentration (10–50%, v/v), and hydraulic retention time (HRT, 24–96 h) were the input parameters. The model showed an R^2 value of 0.91 and, under optimum conditions (14.23 g/L substrate concentration (SC), 32.68% inoculum concentration (IC), and 62.77 h HRT), a hydrogen yield of 12.76 mL H_2 per gram of fermentable sugar (g^{-1} FS) was obtained, which was 2% higher than the predicted yield. A semi-pilot scale-up at 8 L, using the optimum values, gave a cumulative volume of 3739.95 mL and a yield of 321 mL H_2 g^{-1} FS of produced H_2. Microbial analysis from the process effluent indicated the presence of hydrogen producing bacteria belonging to *Clostridium* sp., *Klebsiella* sp., and *Enterobacter* sp.

A techno-economic analysis was performed for large-scale production of hydrogen. The simulated plant had a capacity of 55×10^4 kg sugarcane leaves/year and produced 4×10^6 L of H_2/year. A unit production cost of \$0.96/L H_2 and a gross margin of 20%, with an annual net profit of $\$6.47 \times 10^5$, were obtained with a selling price of \$1.2/L. Sensitivity analysis suggested a decrease in unit production cost as the plant capacity increased, inferring an economy of scales.

This study demonstrated that sugarcane leaf wastes, which are usually burnt prior to harvest, contain sufficient fermentable sugar, which is recoverable through appropriate HCl-based pretreatment, to use as low-cost feedstock for bioprocesses. Biohydrogen production on this feedstock was significantly enhanced by optimizing the key operational parameters, and the process scalability was demonstrated. Furthermore, the techno-economic analysis provided data for strategic R&D investment and early stage knowledge of the economic viability of biohydrogen production from this waste.

Keywords: lignocellulosic biomass, sugarcane leaves, biohydrogen production, techno-economic analysis, biofuel

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Thank you, Mum and Dad, For Everything!

In spite of the fact that just my name appears on the front page of this thesis, a considerable number of individuals have added to its creation. I owe my gratitude to every one of those individuals who have made this thesis conceivable and as a result of whom my postgraduate experience has been one that I will forever cherish!

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Finally, I acknowledge the National Research Foundation for their financial support.

"*We shall require a substantially new manner of thinking if mankind is to survive."*

Albert Einstein

Table of contents

List of figures

Page

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of tables

Chapter 5

List of abbreviations

CH_4…	Methane
CO_2…	Carbon dioxide
COD…	Chemical oxygen demand
DNA…	Deoxyribonucleic acid
FS…	Fermentable sugar
hr…	Hour
H_2…	Hydrogen
HRT…	Hydraulic retention time
OLR…	Organic loading rate
PCR…	Polymerase chain reaction
rpm…	Revolutions per minute
VFA…	Volatile fatty acids

Chapter 1

General Introduction

1.1 The need for renewable energy sources

Sustainable and environmentally clean energy carriers have become the focus in both the energy and environmental sectors. At present, the energy market is dominated by fossil fuels such as oil, coal, and gas (Ghimire et al., 2015). The world population is estimated to increase by 2.38 billion in the next 35 years, and Africa contributes to 34% of this population rise (United Nations, 2015). At the current and projected rate of demand, the depletion of these fuel reserves is inevitable, which will be completely exhausted in the coming decades (Shahriar and Topal, 2009). Increasing populations consume more energy, and therein lies the population–energy dependency cycle, as illustrated in Figure 1.1 (Zabel, 2009).

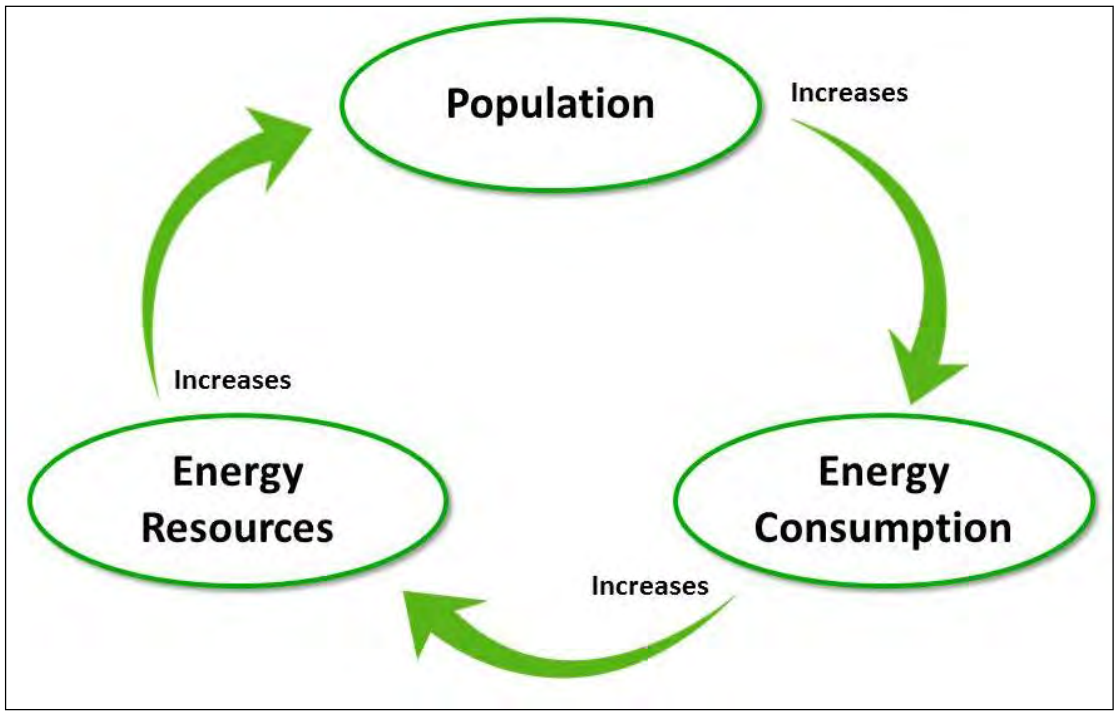

Fig. 1.1. Population and energy dependency (adapted from Zabel, 2009).

In 2006, the total fossil fuel reserves was estimated to be made up of 17.67%, 64.99%, and 17.34% oil, coal, and gas respectively. This equates to 35, 107, and 37 years of usage for oil, coal, and gas, respectively, before depletion, thereby indicating that coal will be the only fossil fuel remaining after

2042 (Shahriar and Topal, 2009). The production of oil between 1990 and 2002 has been relatively constant at 1000 billion barrels per year, but this trend has had an exponential increase over the last decade to approximately 1350 billion barrels per year (Shahriar and Topal, 2009). Figure 1.2 illustrates the global oil production status. A major portion of oil reserves (based on land mass) have clearly reached peak production and have now entered a lull in production (Strahan, 2013), thus acting as precursor to a looming energy crisis. Owing to the uncertainty surrounding the supply of fossil fuels, the price of oil is estimated to increase from USD $56 per barrel in 2015 to $141 per barrel in 2040 and the price of natural gas will increase from $3.73 per British thermal unit (Btu) in 2013 to $7.85 per Btu in 2040 (Energy Information Administration, 2015).

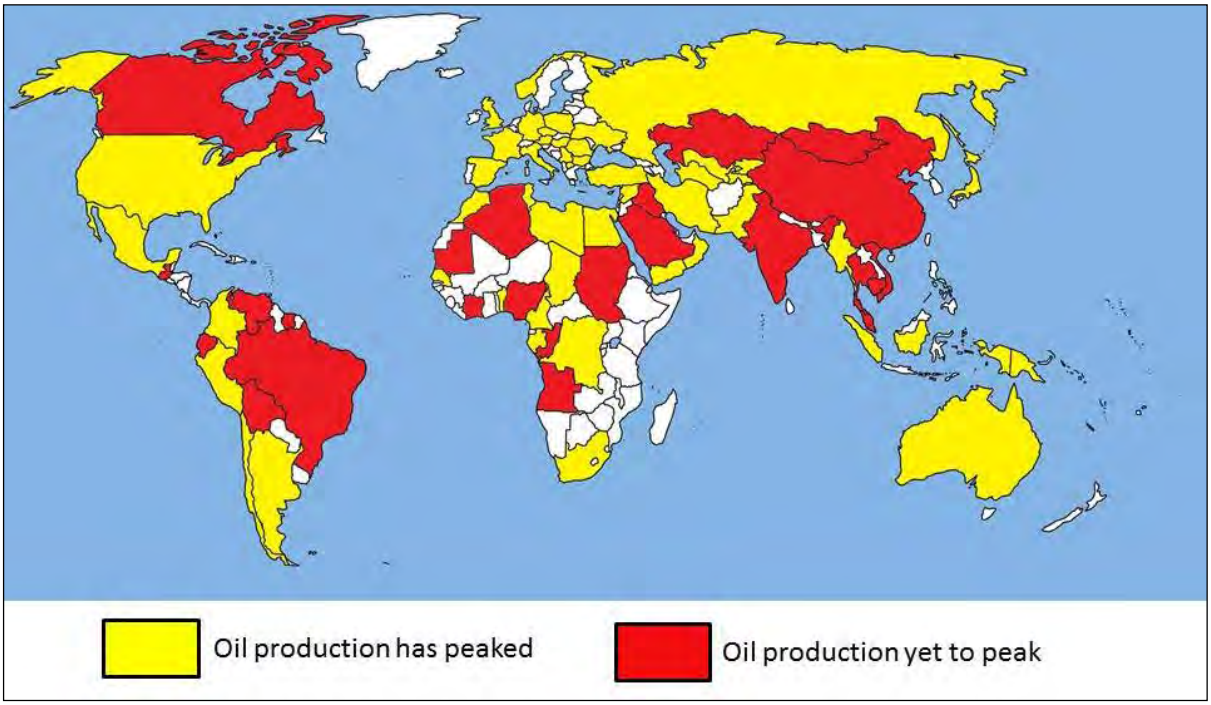

Fig. 1.2. Global nation-wise oil reserves (peaked and yet to peak) (adapted from Strahan, 2013).

Furthermore, it is widely accepted that increasing carbon dioxide (CO_2) levels are directly linked to fossil fuel combustion. This contributes to greenhouse gas emissions that have adverse effects on climate changes (Kothari et al., 2012). Greenhouse gases are mainly composed of CO_2, methane, and nitrous oxide (Stern, 2008). Several studies indicate anthropogenic activity being the root cause of this phenomenon (Ghimire et al., 2015; Huang et al., 2012; Ming et al., 2014). This is perhaps the most pressing issue facing humanity, because it will have disastrous consequences on the environment, human health, and the global economy (Ming et al., 2014). Climate systems are undergoing major changes, which is evident in the widespread melting of snow, rising in sea level, and instability of the

permafrost (Huang et al., 2012). In response to climate changes, plants have been found to contain uncharacteristic phenologies that severely impact ecological processes as well as the agriculture and forestry sectors, consequently affecting food security (Guo et al., 2015). The World Health Organization estimates 250,000 additional deaths per year resulting from the effects of climate change such as malnutrition and heat stress (World Health Organization, 2015). This has expedited the exploration of clean sustainable energy technologies.

Renewable energy comprised 19% of the total energy sector in 2011 (Fig. 1.3) compared with 16% in 2009. There has been a steady increase in renewable energy consumption over the last few years, indicating a growing trend (Renewables, 2011). Biofuels comprise only 0.8% of the total renewable energy cluster, with solar, geothermal, hydropower, and wind being the remaining fractions. However, in recent years, there has been an upsurge in global biofuel production from 0.3% in 2006 to 0.8% in 2011 (Renewables, 2011), suggesting that the energy market is expanding toward biofuels. Sub-Saharan Africa is estimated to have unused land that has the bioenergy capacity of 317 exajoules per year by 2050, i.e., higher than other regions globally (Smeets et al., 2007, Lynd et al., 2015). Biogas in particular has a market potential of 1.1 billion USD in South Africa and could generate 2.5 gigawatts of electricity (Da Silva, 2013).

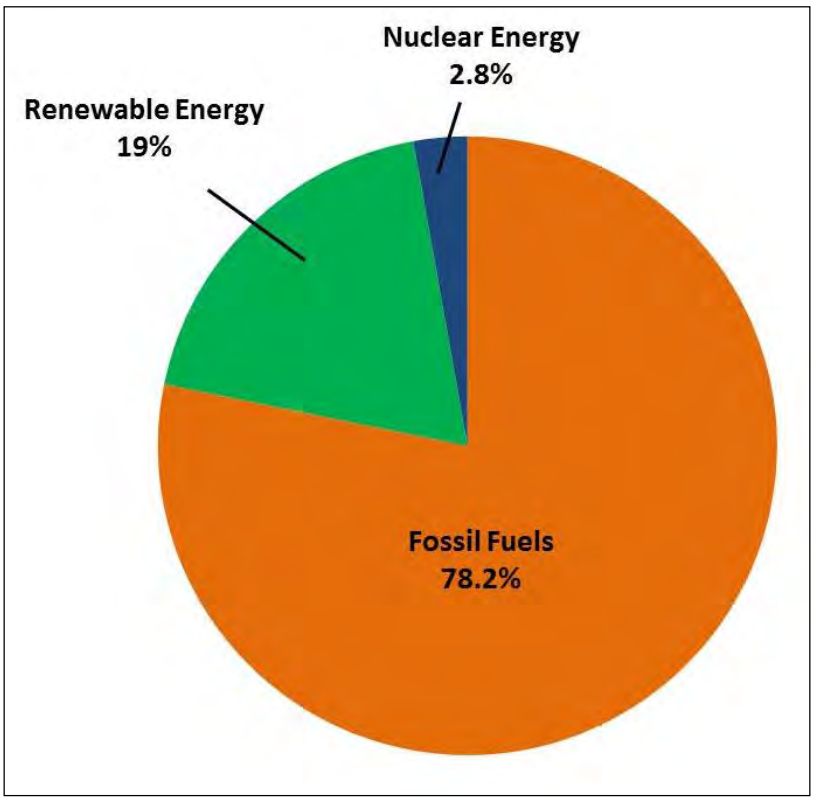

Fig. 1.3. Global energy distribution in 2011 (adapted from Renewables, 2011).

1.2 Hydrogen as a feasible energy source

Hydrogen (H_2), a carbon-free and environmentally friendly fuel, is attracting significant interest owing to its highly versatile applications and high energy content (122kJ/g, i.e., 2.75 times greater than conventional hydrocarbon fuels) (Faloye et al., 2014, Ghimire et al., 2015). It is considered to be a non-polluting alternative to fossil fuels, because water is the only by-product of its combustion. In addition, hydrogen is increasingly being considered over methane, because it encompasses wider industrial applications, such as the use in ammonia synthesis and the hydrogenation of oil, petroleum, and coal (Kothari et al., 2012). Furthermore, hydrogen can be used directly in combustion engines or for electricity production using fuel cell technologies (Alves et al., 2013). Top-tier automotive companies have announced plans to incorporate fuel cells into their vehicles. In response, the US Department of Energy launched H2USA, an agency dedicated to addressing the challenges facing hydrogen infrastructure (U.S. Dept. of Energy (b), 2013).

In 2013, 50 million metric tons of hydrogen was produced worldwide using various methodologies (U.S. Dept. of Energy (a), 2013), as illustrated in figure 1.4. The production of hydrogen can be achieved by electrolysis; however, this is a costly process (U.S. Dept. of Energy (a), 2013). Steam reformation is another approach, though this process uses non-renewable resources, such as methane and coal, thus underscoring its limited supply (Ghimire et al., 2015). Therefore, it is beneficial to develop an economical and sustainable strategy to produce hydrogen. To achieve this objective, hydrogen production using biological processes is being extensively examined (Lay, 2000; Moodley and Kana, 2015; Lin et al., 2012; Cui et al., 2010). The biological production of hydrogen can be divided into either dark fermentation or photo-fermentation. Dark fermentation can produce hydrogen independent of sunlight and is therefore preferred over photo-fermentation, thus enhancing the economics and productivity (Azwar et al., 2014). In addition, dark fermentation can be carried out at ambient temperatures and pressures while using mixed microbial consortia to degrade a variety of waste materials (Ghimire et al., 2015).

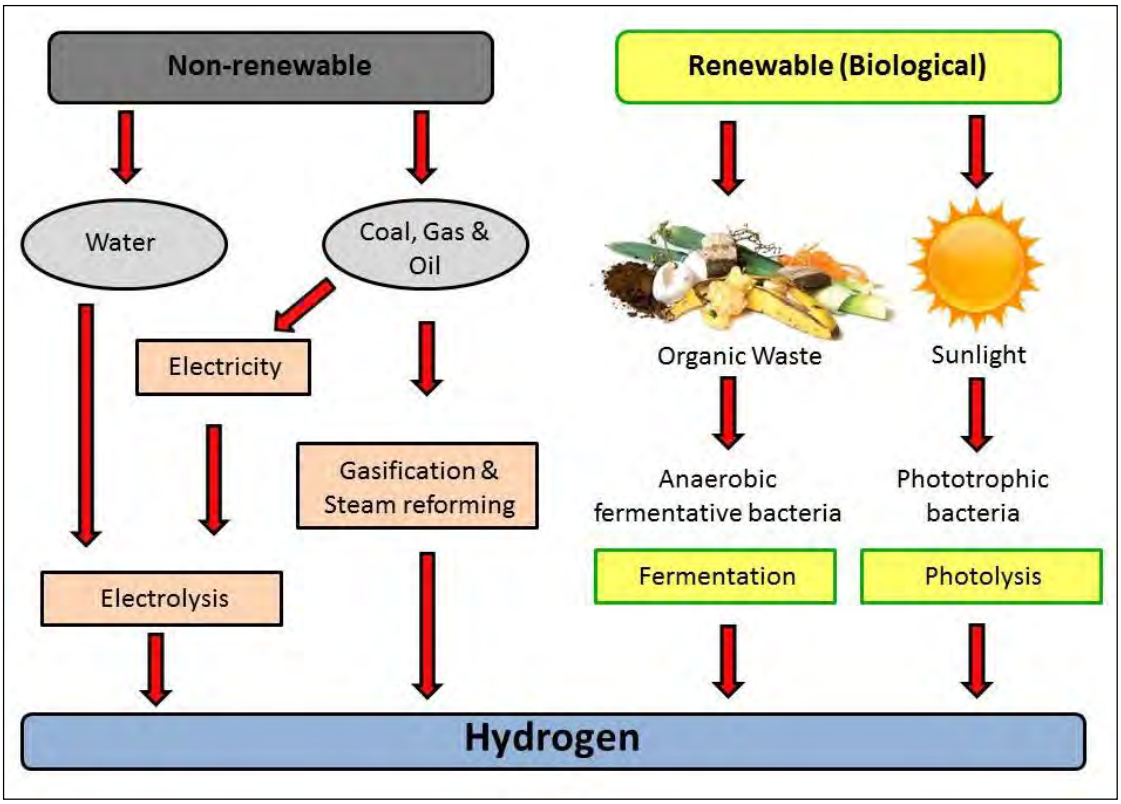

Fig. 1.4. Hydrogen production pathways using renewable and non-renewable resources (Adapted from Luo, 2007).

1.3 Problem statement

Depleting fossil fuel reserves, escalating fuel prices, and climate change are issues of paramount importance facing humanity (Kothari et al., 2012). Biohydrogen production possesses the potential to supplant current production methods, which have limited lifespans. Nonetheless, a challenge facing this process is finding a cheap renewable feedstock and examining the conversion yield of the feedstock (Serra and Zilberman, 2013). Lignocellulosic biomass is considered to be an economical and viable feedstock for biohydrogen production owing to its abundance and high sugar content (Kumar et al., 2015). The lignocellulosic residue of sugarcane, which is cultivated worldwide, amounts to 1.6 billion tons (Sugarcane.org, 2015). The leaf component (comprising 40% of the plant) is regarded as waste and burnt pre-harvest (Smithers, 2014). Large amounts of CO_2 and polycyclic aromatic hydrocarbons are released during the process (Silva et al., 2010); therefore their disposal could cause severe health concerns. This lignocellulosic feedstock can potentially be channeled for renewable biofuel production. A major obstacle in the commercialization of biohydrogen production processes is the low yield on pure sugar substrates (Hallenback and Ghosh, 2009). To overcome this, several strategies can be employed such as: the use of low-cost lignocellulosic-based feedstock, the development of efficient and low-cost pretreatment technologies for the lignocellulosic materials, and the optimization of key fermentation parameters and the scale up studies.

1.4 Aims and objectives

The aim of this study was to optimize the production of fermentable sugars namely xylose and glucose from sugarcane leaves using various acid-based pretreatment models, optimize the physico-chemical parameters for biohydrogen production from these fermentable sugars, and undertake a techno-economic analysis for a large scale plant biohydrogen production using waste sugarcane leaves.

The following specific objectives were carried out:

i. Modeling and optimizing three models for the production of xylose and glucose from waste sugarcane leaves using different acid-based (HCl, H_2SO_4, and HNO_3) hybrid pretreatments

ii. Modeling and optimizing the hydrogen response on the operational parameters of the substrate and inoculum concentrations and Hydraulic Retention Time using the optimally pretreated substrate mention in objective (i).

iii. Assessment of the semi-pilot scale production of biohydrogen using the substrate obtained in objective (i) and the optimized operational conditions derived from objective (ii).

iv. A techno-economic analysis of a large-scale production of biohydrogen from pretreated waste sugarcane leaves using dark fermentation.

1.6 Outline of dissertation

This thesis comprises four chapters presented in research paper format. Each experimental chapter is self-contained, containing an introduction, materials and methods, results and discussion, conclusion, and references. The description, assessment and application of utilizing waste sugarcane leaves for the production of biohydrogen are central to all chapters.

Chapter 2 presents an overview of sugarcane leaves as a potential feedstock for biohydrogen production. It examines the pretreatment methodologies for lignocellulosic biomass as well as the potential for producing hydrogen from sugarcane leaves via dark fermentation.

In Chapter 3, three acid-based pretreatment models namely HCl, H_2SO_4, and HNO_3, are optimized for production of xylose and glucose from waste sugarcane leaves. The pattern of release of xylose and glucose using these pretreatment models is assessed using the response surface graphs.

Chapter 4 focuses on the optimization of key operational parameters for biohydrogen production from waste sugarcane leaves. The optimum setpoints are determined and validated experimentally. A semi-pilot scale production process using the optimized operational conditions is assessed.

In Chapter 5, a techno-economic analysis of biohydrogen production from waste sugarcane leaves in a 55×10^4 kg sugarcane leaves/year capacity plant is carried out.

Chapter 6 integrates the findings from the experimental chapters and provides conclusions from this research. Recommendations for future research prospects are also provided.

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Chapter 2

Waste sugarcane leaves as a potential feedstock for biohydrogen production

2.1 Abstract

Microbial production of biohydrogen is projected to be a key commodity in sustainable energy in the future, especially when it is produced in an economically friendly process. Lignocellulosic biomass such as sugarcane leaves is considered a suitable feedstock for biohydrogen production since it is rich in carbohydrates and available at low cost. An estimated 640 million tons of waste sugarcane leaves are generated annually and burnt prior to cane harvest. This results in the emission of carcinogenic particles and escalated amounts of carbon dioxide. This review examines pretreatment methodologies for lignocellulosic biomass as well as the potential for producing hydrogen from sugarcane leaves via dark fermentation since it is a promising approach for high yields. In addition, key operational parameters and economics are discussed.

Keywords: Sugarcane leaves, biohydrogen production, biomass pretreatment, dark fermentation

2.2 Introduction

A key issue confronting humankind is energy security and the optimal use of natural resources (Okudoh et al., 2014). Fossil fuels remain the major source of energy and are expected to meet global demand until 2030. Oil and gas demand were expected to increase from 36 million barrels per day in 2006 to 46 million barrels per day in 2015 while accelerating to 61 million barrels per day by 2030 (Shahriar and Topal, 2009). Regardless of supply, once extraction costs increase, renewable energy becomes a more lucrative option. The Food and Agriculture Organization (FAO) reports that Africa has the lowest gross domestic product (GDP) ($1629.5 billion) compared with other regions worldwide. A major portion of African nations' budgets are spent on energy imports rather than energy development (Okudoh et al., 2014); this echoes the need for energy independency.

Escalating atmospheric CO_2 concentrations, the majority originating from tailpipe emissions, are regarded as the main contributing factor to greenhouse gas (GHG) emissions (Zhang et al., 2015, Chaubey et al., 2013). A fundamental goal in any renewable energy strategy is to mitigate GHG emissions and alleviate the effects of environmental degradation. The effects of the current concentration of CO_2 in the atmosphere will be felt for hundreds of years, even if CO_2 emissions stopped completely today (Frolicher et al., 2014). This indicates the urgent need for a clean source of energy.

The high demand for non-food-based feedstocks has created a paradigm shift in the need to exploit sustainable and less expensive resources for their bioconversion into value-added bio-products (Chandel et al., 2011). Agricultural industries generate millions of tons of lignocellulosic waste annually that have the potential to serve as low-cost feedstock for energy production. Unlike first generation biofuels that are generated from crops such as canola, soy, corn, and sunflower, which exacerbate food insecurity, second generation biofuels are derived from lignocellulosic waste biomass. This is a more attractive process since it uses low-cost materials while not displacing food crop production (Smith et al., 2013). Sugarcane is an agricultural crop that is cultivated worldwide with an annual production of 1.6 billion tons (Chandel et al., 2011). The world's sugar is primarily obtained from sugarcane, one indication of its economic importance as an agricultural crop. Sugarcane is mainly produced as a food crop, although some countries are exploring energy routes (Smithers, 2014). Sugarcane bagasse and leaves, which have significant potential in biotechnological applications, comprise a large percentage of the annual production yield (Smithers, 2014). The bio-conversion of biomass to energy will lessen fossil fuel dependency while mitigating environmental impacts (IPCC, 2007). The use of lignocellulosic biomass has been reported (Chen et al., 2012, Magnusson et al., 2008, Han et al., 2012, Moodley and Kana, 2015).

This review focuses on the potential of using sugarcane leaves (SCL) for biohydrogen production via dark fermentation. The pretreatment of lignocellulosic material and operational parameters affecting dark fermentation are also discussed. Finally, the economic viability of biohydrogen production from SCL is presented.

2.3 Sugarcane production in South Africa

Sugarcane (*Saccharum officinarum*) is categorized as a tall grass with a large stem (Fig 2.1) and is mainly cultivated in tropical countries (Contreras et al., 2009). In 2011, 1.6 billion tons of sugarcane was produced in approximately 67 countries, which accounted for 22.4% (by weight) of global agricultural production (Chandel et al., 2011; Chauhan et al., 2011). Sugarcane is an essential crop that meets the basic demands of the human body; therefore, it is an integral component to human life (Chauhan et al., 2011). It currently supplies 70% of the global sugar demand, with other crops such as cassava and sugar beets providing the remaining 30% (Contreras et al., 2009). The major sugarcane producing countries (Fig 2.2) are reported to be Brazil and India, with a combined production of approximately 558 million tonnes during the 2009-10 season (Chauhan et al., 2011). South Africa, in particular, produces, on average, 20-22 million tonnes annually (Smithers, 2014).

The South African sugar industry is worth R12 billion ($846 million) and is regularly ranked in the top 15 of 120 sugar-producing countries (SASA, 2015). The sugar industry creates almost 85,000 jobs for an estimated 35,000 people. These factors contribute to South Africa's GDP and overall economy (Deressa et al., 2005). In South Africa, approximately 430,000 ha are used to grow the 20-22 million tons of sugarcane annually, which are processed at 15 sugar-producing factories (Smithers, 2014). The sugarcane is grown in both steep and flat terrain and cultivated under dry and irrigated land (Smithers, 2014). The harvesting period usually runs from April to December, with over 90% of harvesting done manually (Meyer and Fenwick, 2003). The 20 million tons of cane biomass represent the energy equivalent of 1.75 million tons of coal, which equates to 1,600 MW of electricity. This biomass could potentially produce 600 MW, which represents 20% of the target of renewable energy established for South Africa (Smithers, 2014).

Figure 2.1. Sugarcane plantation

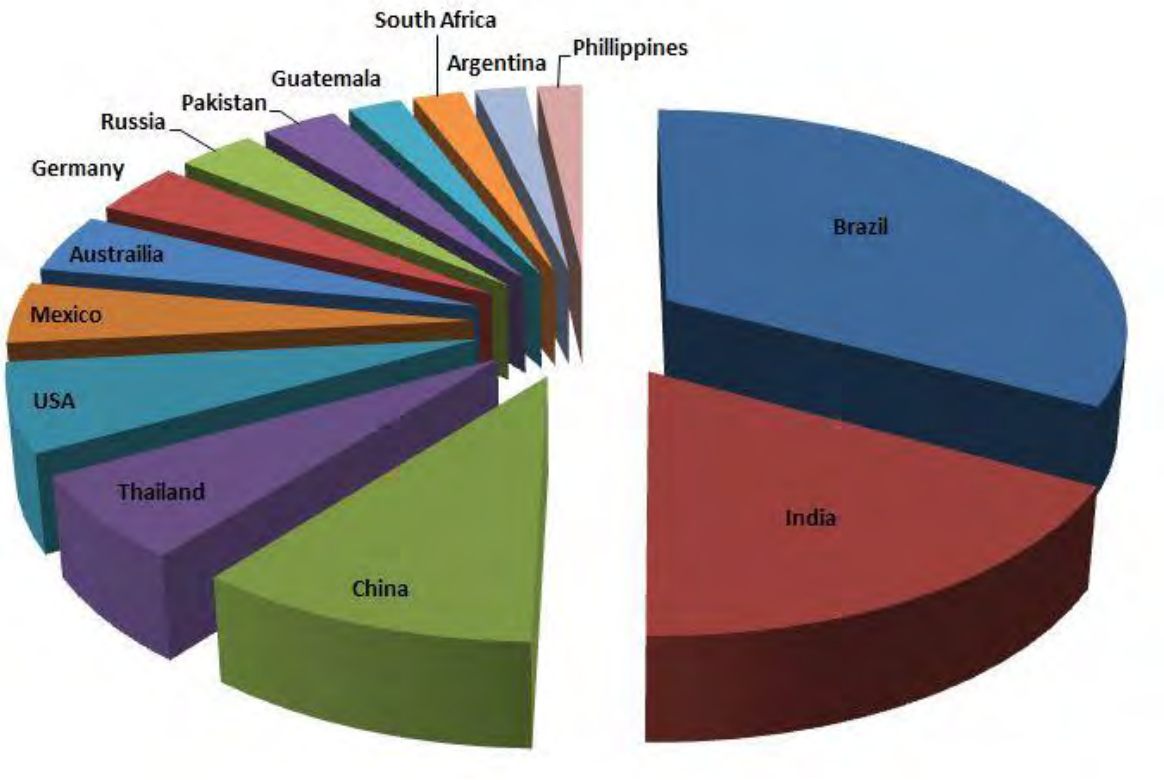

Figure 2.2. Major sugarcane producing countries between 2009-10 (Adapted from Chauhan et al., 2011)

The sugarcane plant consists of the stalk and the leaves (Chandel et al., 2011). The stalk contains the sugar that is recovered during production. It is crushed and the juice extracted with water. The remaining fibrous tissue, called bagasse, is either discarded or burnt to supply the energy requirements of the mills (Smithers, 2014). The leaves, which have a high calorific value, remain largely unexploited and are thus discarded as waste and burnt prior to harvest.

Sugarcane leaves (SCL) are organically rich in carbohydrates, with the largest fraction being cellulose and hemicellulose. The composition of dry SCL is illustrated in Fig 3 and the sugar composition of SCL is shown in Table 1. It also contains glucan and xylan (approximately 33 and 18% respectively); inferring high concentrations of glucose and xylose which could potentially be extracted through appropriate pretreatment.

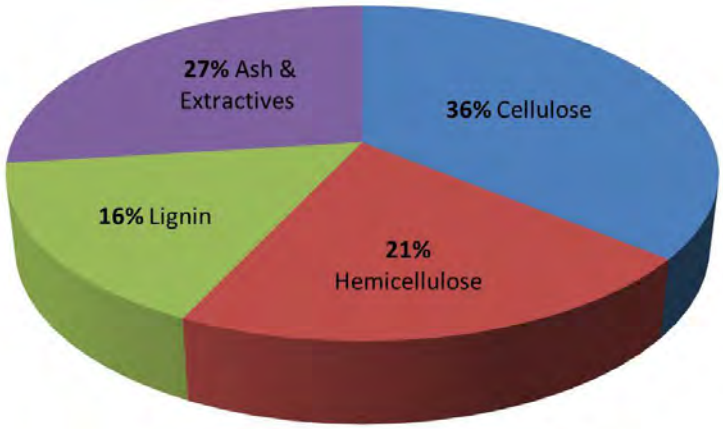

Figure 3. Lignocellulosic composition of sugarcane leaves (Adapted from Eggleston et al., 2014; Rocha et al., 2014)

Table 1. Sugar composition of sugarcane leaves (Ferreire-Leitao et al., 2010)

Component	Percentage
Glucan	33.3
Xylan	18.1
Arabinan	3.1
Galactan	1.5
Mannan	1.5
Lignin	36.1
Total	**93.6**

2.4 Challenges of sugarcane leaves disposal

Sugarcane harvesting is often challenged by sharp leaves that make manual harvesting difficult (Jutakanoke et al., 2012). In addition, some terrains prevent the use of mechanical harvesting equipment due to poor soil quality (Smithers, 2014). To overcome this, the sugarcane field is frequently burnt prior to harvest to facilitate the process. Sugarcane leaves, which constitute almost 40% of the plant, are completely destroyed in this process thereby eliminating their feedstock potential (Meyer and Fenwick, 2003; Smithers, 2014). In addition to the loss of biomass, it also poses severe environmental risk as a result of carbon emissions.

Carbon dioxide emissions are estimated to be 0.881 ton CO_2/ha during cane burning in Brazil (Capaz et al., 2013) accompanied by the release of large amount of soot. This soot contains carcinogenic polycyclic aromatic hydrocarbons (PAHs), carbonyl compounds, and volatile organic compounds as shown in Table 2 (Hall et al., 2012). Some of these compounds are found naturally in trace quantities in the atmosphere; however, Silva et al. (2010) reported 5 times more PAHs in the atmosphere during harvesting season compared with non-harvesting season. Lung cancer pathogenesis and other respiratory diseases have been linked to the prolonged exposure to PAHs (Silva et al., 2010).

For this reason, by 2018, cane burning will be phased out in Brazil where mechanical harvesting is feasible (Leal et al., 2013). Moreover, to alleviate environmental degradation, some studies have proposed the use of these sugarcane leaves as a feedstock for bioenergy production (Moodley and Kana, 2015, Jutakanoke et al., 2012).

Table 2. Summary of (polycyclic aromatic hydrocarbons) PAH emission factors from sugarcane leaf burning (mg/kg) (Hall et al., 2012)

PAH	Sugarcane	
	Dry leaves	Whole stalks
Naphthalene	4.83 ± 0.72	5.24 ± 2.45
Acenaphthylene	0.78 ± 0.09	0.80 ± 0.30
Acenaphthene	-	0.11
Fluorene	0.26 ± 0.05	0.27 ±0.20
Phenanthrene	0.73 ± 0.10	0.87 ± 0.25
Anthracene	0.14 ± 0.03	0.15 ± 0.06
Fluoranthene	0.20 ± 0.02	0.30 ± 0.05
Pyrene	0.18 ± 0.01	0.27 ± 0.05

-: Data not available

2.5 Biohydrogen from agricultural wastes

Lignocellulosic biomass is the most abundant material on earth with an annual yield of 200 billion tons (Khamtib et al., 2011). Lignocellulose contains polymers rich in fermentable carbohydrates that can be bio-converted microbially into hydrogen (Zheng et al., 2014). They are the most low-cost and easily available organic waste that can be converted into biohydrogen due to their biodegradability (Guo et al., 2010). Biohydrogen from lignocellulosic residues have been vastly reported (Chen et al., 2012; Guo et al., 2010; Han et al., 2012; Lay, 2000; Magnusson et al., 2008; Moodley and Kana, 2015; Saraphirom and Reungsang, 2010) with yields that ranged from 24.8 to 60.2 ml H_2/g feedstock.

Although biohydrogen from agricultural waste is an attractive source of energy, there are still many obstacles to overcome before its commercialization can be realized. One of these challenges is the low yields obtained. The digestibility of lignocellulosic biomass is often hampered by the recalcitrance of the polymer matrix, thus affecting the hydrogen yield. Furthermore, the presence of methanogens and other hydrogen consuming bacteria in mixed inoculum severely hamper hydrogen yields. In addition, operational parameters are key factors in determining the metabolic pathways of hydrogen production (Ghimire et al., 2015). To overcome these challenges, various strategies have been developed. These include biomass pretreatment, inoculum pretreatment and process optimization.

2.6 Improvement strategies for biohydrogen production from agricultural residues

2.6.1 Feedstock pretreatment

Lignocellulosic biomass is composed of three basic polymers; cellulose, hemicellulose and lignin. Cellulose and hemicellulose are carbohydrate rich thus fermentable after appropriate pretreatment (Zheng et al., 2014). Cellulose, the largest component, is made up of glucose disaccharides linked by β-1,4 glycosidic bonds and the hydroxylic groups which are linked by hydrogen bonds. This cross-linking between the cellulose chains results in a rigid crystalline structure that is highly recalcitrant towards biological degradation (Ha et al., 1998, Behara et al., 2014).

In contrast, hemicellulose is vastly different. It is composed of various pentoses (xylose and arabinose), hexoses (glucose, galactose, mannose), and acids (glucuronic, methyl glucuronic and galacturonic acid) arranged in short branched chains. The branched characteristic of hemicellulose makes it highly susceptible to biological and chemical hydrolysis (Ademark et al., 1998, Zheng et al., 2014).

Lignin is a complex aromatic heteropolymer made up of phenylpropanoid precursors (Mood et al., 2013). Lignin is integral to the cross-linking between cellulose and hemicellulose and forms the rigid

cell wall. Lignin is insoluble in water and dissolves in temperatures above 180ºC which makes it the most obstinate part of the plant cell wall (Zheng et al., 2014).

Sugarcane leaf (SCL) pretreatment is essential in order to remove lignin and hemicellulose and to disrupt the crystalline structure of cellulose thereby making the monomeric sugars available for further processing (Behara et al., 2014). Pretreatment works by either increasing the surface area or porosity of the biomass to remove lignin, hemicellulose, and also reduce cellulose crystallinity as illustrated in Fig 4 (Zhang et al., 2009). SCL pretreatment is crucial to facilitate the enzymatic hydrolysis and subsequent bioconversion to biofuels. (Behara et al., 2014). An effective pretreatment regime should preserve and decrystallize the cellulose and depolymerize hemicellulose; reduce inhibitor formation, which impedes enzymatic hydrolysis; require low energy; and be economically friendly (Chaturvedi and Verma, 2013).

Some of the pretreatment methods that have been reported for lignocellulosic feedstock are further discussed below:

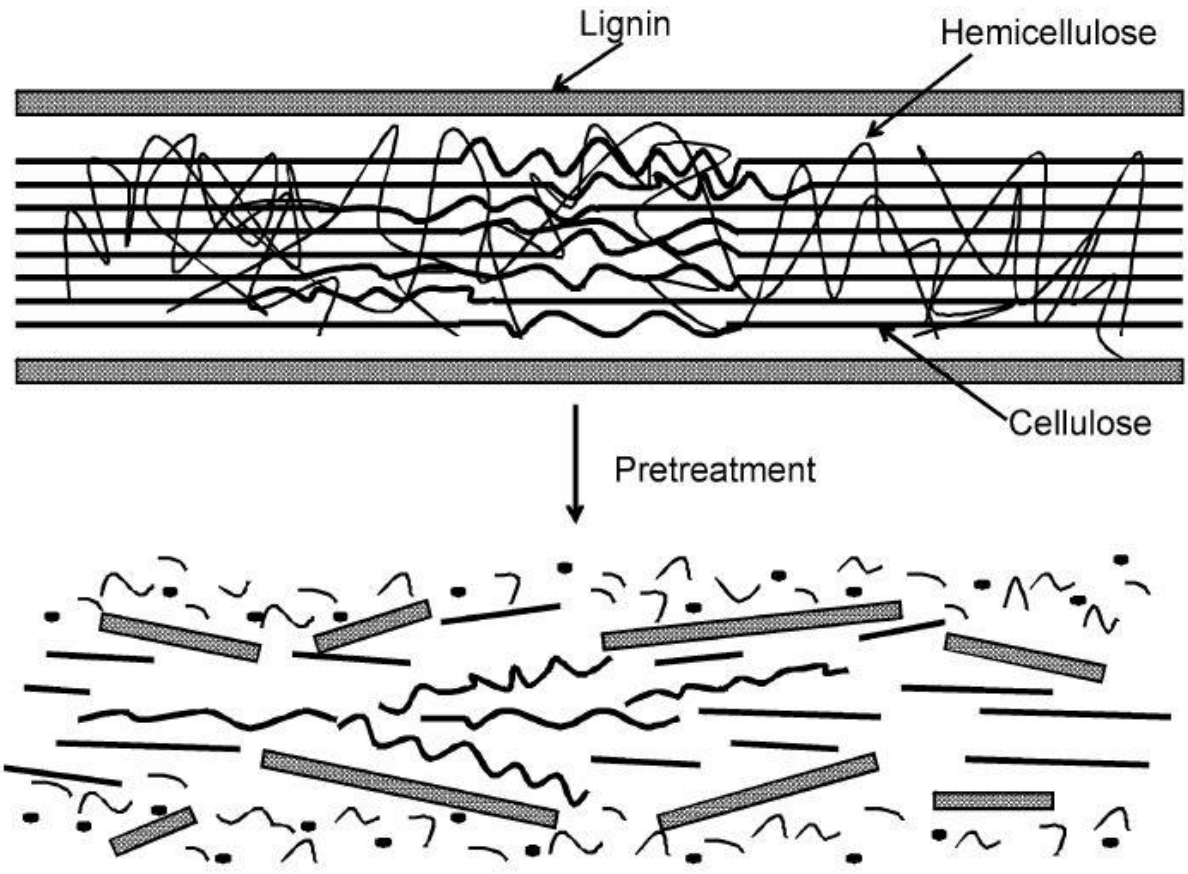

Figure 4. Effect of pretreatment on lignocellulose (Chaturvedi and Verma, 2013)

2.6.1.1 Physical techniques

Physical pretreatment techniques are employed to increase the surface area and pore size of biomass. This technique includes comminution (milling and grinding), liquid hot water pretreatment, extrusion, and irradiation (Zheng et al., 2014). Comminution is used to reduce particle size and is often coupled with another pretreatment.

Particle size reduction alters the intrinsic structure of lignocellulose, thus increasing accessibility for either chemical or biological pretreatment (Kratky and Jirout, 2011). Kivaisi and Eliapenda (1994) reported a 30% improvement in methane yield by reducing the particle size of bagasse and coconut fibres from 5 mm to 0.85 mm. In another study, biogas production from agricultural waste was increased by 6% with a decrease in particle size from 30 mm to 0.088 mm (Sharma et al., 1988).

Extrusion helps to physically and chemically alter the lignocellulosic material by employing mixing, heating and shearing forces (Yoo et al., 2011). For instance, a 132% increase in glucose yield was obtained from soybean hulls when pretreated with extrusion (Yoo et al., 2011). The pretreatment of rice straw with extrusion was shown to increase methane production from 38.9% to 59.9% (Zhang et al., 2015).

Similarly, liquid hot water is frequently employed in both the pulp and bioethanol industries as a pretreatment agent. It is a process in which biomass is treated in water at a high temperature and pressure without the use of chemicals (Rogalinski et al., 2008). During this process, water infiltrates the cell, hydrates cellulose, and solubilizes hemicellulose and partially removed lignin (Zheng et al., 2014). Sugarcane bagasse subjected to liquid hot water pretreatment gave a total reducing sugar yield of 26.50 g/L, which is 12% higher than acid treatment (Timung et al., 2015).

Irradiation is another physical technique commonly used for lignocellulosic feedstock pretreatment. This technique entails the use of microwave, ultrasound, gamma-ray or electron beams. Microwave is most commonly employed since it generates rapid heat with reduced thermal gradients (Zheng et al., 2014). This process disrupts the lignocellulosic structure to enhance the enzymatic treatment (Mood et al., 2013). In a study by Shahriari et al. (2012*)* microwave pretreated organic fraction municipal solid waste showed a 4-7% improvement in biogas production.

2.6.1.2 Chemical techniques

Chemical pretreatment refers to the use of acids, bases or ionic liquids to modify the properties of lignocellulosic biomass. Among the various pretreatment strategies, chemical pretreatment is the most researched and extensively used for delignification (Zheng et al., 2014). This includes acidic and alkaline-based pretreatments.

Sulfuric acid pretreatment is the most popular method for hydrolysing polysaccharides into monosaccharides. For instance, coastal Bermuda grass pretreated with 1.2% sulphuric acid at 140^{o}C for 30 min produced 94% of theoretical sugar yield (Redding et al., 2011). Other commonly used acids include nitric acid and phosphoric acid. Another study reported a 78% reduction in cellulose crystallinity with the use of dilute phosphoric acid on wheat bran (Nair et al., 2015). Martinez et al. (2015) examined both acid and alkaline pretreatment and observed a 50% loss in xylan under alkaline conditions and zero loss with the acidic pretreatment. Concentrated acids are more economical, since lower temperatures are required compared to low concentrated acid (Taherzadeh and Karimi, 2008). Some major challenges associated with acidic pretreatment are corrosion of equipment, toxicity, acid recovery and the degradation of some monomeric sugars into furan-type inhibitors (Mood et al., 2013).

Similar to acidic pretreatment, the use of alkaline agents for the removal of lignin can increase the accessibility for microbial or enzymatic degradation (Chaturvedi and Verma, 2013). However, hemicellulose and cellulose solubilize to a lesser degree with alkaline pretreatment (Mood et al., 2013). In particular, ester linkages are broken thereby separating xylan residues (Sun and Cheng, 2002).

Sodium hydroxide (NaOH) is the most frequently used alkaline pretreatment agent and functions as a saponifying agent by cleaving lignin-carbohydrate linkages (Zheng et al., 2014). For example, soybean straw pretreated with NaOH achieved 46.37% xylan removal (Wan et al., 2011). Zheng et al. (2009) also reported a 79% reduction in lignin, cellulose and hemicellulose content for corn stover pretreated with 2% NaOH. The effect of lime (calcium hydroxide) on rice hulls was studied by Saha and Cotta (2008). A total of 12.6% of total sugars was recovered using 100 mg lime/g rice hulls at 121^{o}C for 1hr and no furfural-type inhibitors was observed. Ammonia fiber explosion (AFEX) pretreatment has also been shown to reduce lignin content and cellulose crystallinity (Chaturvedi and Verma, 2013). This process uses liquid ammonia at high temperatures and pressure for a specific period following a sudden reduction in pressure. AFEX has several advantages over other treatments since it does not form toxic products, does not require size reduction, and yields 99% sugar recovery (Behara et al., 2014). AFEX treatment of corn stover showed a 52% improved enzymatic digestibility

compared to untreated material (Bals et al., 2010). Another study showed a 67.8% improvement in hydrogen yield from AFEX pretreated wheat straw (Cao et al., 2013).

Ionic liquids (ILs) are a new category of solvents with high polarities, low melting points, wide liquid temperature range, and thermal stability (Behara et al., 2014). Cellulose is fragmented through the oxygen and hydrogen atoms from the cellulose hydroxyl groups and forms electron donor-acceptor complexes which interact with ILs. This interaction causes breakages in the cellulose chains (Feng and Chen, 2008). Bagasse pith pretreated with an ionic liquid (1-butyl-3-methylimidazolium chloride) at 120^{o}C for 30 min yielded 92.3g/l glucose (Wang et al., 2015). Another study reported a 90% conversion of cellulose to glucose from mixed softwoods treated with the same IL (Trinh et al., 2015). Table 3. summarizes various studies that employed chemical pretreatments.

Table 3. Commonly employed pretreatment techniques for lignocellulosic biomass

Feedstock	Pretreatment	Conditions	Yield	Ref
Jatropha curcas	Acidic	1.5% H_2SO_4, 136 ^oC, 30 min	80% cellulose conversion	Garcia et al., 2014
Rapeseed straw	Acidic	0.32% H_2SO_4, 202 ^oC, 5 min	41.1 g sugar/ 100g	Lopez-Linares et al., 2013
Sugarcane bagasse	Acidic	2-6 % H_2SO_4, 100-128 ^oC, 0-300 min	21.6g/l xylose, 3g/l glucose	Aguilar et al., 2002
Rice hulls	Acidic	1.3% H_2SO_4, 202 ^oC, 33 min	84% conversion of glucans	Dagnino et al., 2013
Napier grass	Alkaline	7 % NaOH, 35 ^oC, 4 hr	7.3 g/l glucose	Liong et al., 2012
Corn stover	Alkaline	Ammonia percolation	80% lignin reduction	Kim et al., 2003
Wheat straw	Alkaline	2.15% H_2O_2, 35 ^oC, 24 hr	8.6 (w/v) reducing sugar	Saha and Cotta, 2006

2.6.1.3 Biological techniques

Biological pretreatment can be performed by fungi, a microbial consortium or via enzymatic hydrolysis (Zheng et al., 2014). This pretreatment technique requires low energy, no chemicals and limited formation of inhibitors; however, these advantages are over-shadowed by the extensive pretreatment time required, thus limiting its commercial use (Taherzadeh and Karimi, 2008). The basis of biological treatment involves microorganisms (such as white-rot fungi) that produce cellulolytic enzymes capable of degrading lignocellulose (Chaturvedi and Verma, 2013). Wang and Li (2012) investigated the pretreatment of agricultural residues with *Ceriporisis subvermispora* and reported a 56% increase in glucose, 3 times higher than untreated material. Rice husk pretreatment by *Phanerochaete chrysosporium* yielded 44.7% total reducing sugar (Potumarthi et al., 2013). Cellulase and hemicellulase are commonly used enzymes to enhance the saccharification of lignocellulosic biomass however, the high costs of these enzymes limits their commercial applications (Zheng et al., 2014).

2.6.2 Inoculum development and pretreatment for biohydrogen production

Biohydrogen production via dark fermentation can be achieved using different inoculum sources: mixed, pure, and co-cultures. Mixed cultures have been frequently reported in the bioconversion of lignocellulosic biomass to biohydrogen. Mixed cultures include animal dung, soil, sewage sludge and wastewater, and are often preferred over pure cultures since they offer operational simplicity, low sterile conditions requirement, and robust adaptability to fluctuating environmental conditions, thus indicating its suitability for scale-up processes (Faloye et al., 2014). However, due to the presence of methanogens, homoacetogens and sulfate reducing bacteria, hydrogen yields are relatively low from mixed cultures (Guo et al., 2010). To combat this, appropriate inoculum pretreatment should be employed (Song et al., 2012).

Inoculum pretreatment normally relies on selecting spore-forming hydrogen producers such as *Clostridium*, since they can withstand the harsh chemical or thermal treatment (Ghimire et al., 2015). Song et al. (2012) reported a hydrogen yield of 290.8 ml/l culture with a microbial community dominated by *Clostridium* sp. and *Enterobacter* sp. Zhang et al. (2015) examined fermentative hydrogen production from corn stover and found facultative anaerobes *Enterobacter* sp. *Klebsiella* sp. and *Citrobacter* sp. present in large numbers. A number of different pretreatment techniques have been used, these include thermal and chemical pretreatment, and the summary is presented in Table 4.

Depending on the substrate and microbial community, the metabolic processes can either favour the acetate (Eq 1) or butyrate (Eq 2) pathway (Hawkes et al., 2007)

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

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

Theoretically, a lower molar of hydrogen (2 mol) is achieved when the butyrate pathway is favoured compared with the acetate pathway (4 mol). A mixed culture usually produces butyrate and acetate in a 3:2 ratio, which could potentially yield 2.5 mol H_2 / mol hexose (Guo et al., 2010).

Hydrogen-producing microorganisms have been isolated from environmental samples and employed as pure cultures. An et al. (2014) reported a hydrogen yield of 2.31 mol/ mol xylose from *Clostridium beijerinckii*. Another study employed *Clostridium bifermentans* and achieved 0.9 mmol H_2/g dried solids (Wang et al., 2003). However, the use of pure cultures has many limitations such as the stringent need for sterile conditions as well as substrate selectively.

Table 4. Inoculum pretreatment for enriching hydrogen producing microorganisms

Inoculum	Pretreatment	H_2 yield	Major H_2 producer	Reference
Anaerobic sludge	Boiled at 100 ^oC for 15 min	1.44 ± 0.01 mol/mol glucose	*Clostridium* sp	Si et al., 2015
Anaerobic digester sludge	Heated at 70 ^oC for 30 min	2.96 ± 0.14 mol/mol hexose	*Clostridium* sp	Nasr et al., 2015
Cow dung	Infrared treatment for 2 hr	2.55 l/l culture	*Clostridium* sp., *Enterobacter* sp., *Bacteroides* sp.	Song et al., 2012
Anaerobic sludge	Heated at 121 ^oC for 10 min	248 ml/g total sugar	-	Moodley and Kana, 2015
Anaerobic sludge	pH 8.9 for 24 hr and heated at 121 ^oC for 15 min	1.35 mol /mol glucose	-	Faloye et al., 2013
Digested sludge	pH 3.0 for 24 hr	221.5 mL/g glucose	-	Wang and Wan, 2008a

-: Data not available

2.6.3 Optimization of process parameters

2.6.3.1 pH

pH is a critical factor that influences fermentative hydrogen production since hydrogenase activity is directly affected (Wang and Wan, 2009). The initial pH prior to fermentation has been shown to directly affect the microbial population and therefore depends on the composition of the microbial community (Mohammadi et al., 2012). For instance, Wang and Wan (2009) observed an increase in hydrogen production with increasing initial pH.

Several studies report the optimum pH for biohydrogen production in the range of 5.5 – 6.7 (Faloye et al., 2013; Moodley and Kana, 2015; Chaganti et al., 2012; Chong et al., 2009). Guo et al. (2010) reported the optimum pH range for organic food waste, lignocellulosic waste and animal manure to be 6.0-7.0, 7.0 and 7.0 respectively; however, conflicting results were observed by Sekoai and Kana (2013). These authors reported an optimum pH of 7.9 for biohydrogen production from agro-municipal waste. This variation may be as a result of the underlying factors such as substrate and inoculum type, and other operating conditions.

Furthermore, the accumulation of volatile fatty acids (VFA) in the effluent is a contributing factor which can decrease pH leading to the inhibition of hydrogenase activity (Guo et al., 2010 pH control is important in enhancing high biohydrogen conversion rates by directly minimizing methanogenic activity (Ghimire et al., 2015). Table 5 summarises optimum pH ranges employed in biohydrogen production processes.

2.6.3.2 Hydraulic Retention Time (HRT)

Hydraulic retention time describes the length of time a specific volume of liquid is retained in the working volume of a reactor. This time period could negatively impact microbial metabolism if it is too long or too brief (Mohammadi et al., 2012). HRT is dependent on the organic loading rate (OLR) and the composition of the substrate (Mao et al., 2015). In batch systems, shorter HRTs are preferred for hydrogen production compared to longer HRTs used in continuous systems. For instance, Shin and Youn (2005) reported that extending the HRT in a semi-continuous system from 2 days to 5 days while reducing the OLR from 10 to 8kg VS/m^3 /d increased the hydrogen yield by more than 50%. In contrast, Sekoai and Gueguim Kana (2014) reported a peak hydrogen fraction (47%) at approximately 36 hr, and a further increase in time drastically declined the hydrogen fraction. Longer HRTs in a batch system can allow for an accumulation of VFAs, which decreases the pH of the system and ultimately hampers the metabolic activity involved in hydrogen production (Mohammadi et al., 2012). Moreover, the complexity of the substrate will influence the biochemical pathways, which directly

affects the HRT. Table 5 summarises some reported optimum HRTs employed in biohydrogen production.

2.6.3.3 Organic Loading Rate (OLR)

Organic loading rate is the biological conversion efficiency of an anaerobic digestion system (Chen et al., 2014) or the amount of volatile solids fed into a digester (Moa et al., 2015). It is a key parameter in biological hydrogen production. Lin and Chang (1999) reported an increase in hydrogen yield of 0.49 ml-H_2 mol^{-1} glucose when the OLR was doubled from 20g COD L^{-1} d^{-1} to 40g COD L^{-1} d^{-1}. Several studies found that an increase in OLR has a positive impact on the yield of hydrogen whereas others reported a negative impact (Tawfik and Salem, 2012). The organic loading rate can also affect the microbial community composition. In a study at lower OLR (2 g COD l^{-1} h^{-1}), more diverse and variable microbial populations (*Selenomonas*, *Enterobacter* and *Clostridium* sp.) were observed while only *Clostridium* spp. were reported at OLR higher than 2 g COD l^{-1} h^{-1} (Luo et al., 2008).

Increasing the OLR can also enhance the production of biogas; however, beyond some limit the productivity and equilibrium of the digestion system can be disturbed (Mohammadi et al., 2012). Bacterial inhibition can also occur at high OLR due to higher acidogenesis which leads to an increase in volatile fatty acid production and ultimately irreversible acidification (Mao et al., 2015). Cheng et al. (2012) reported that xylose at lower concentrations (1% w/v) resulted in a higher yield of hydrogen (190 ml/g xylose) compared with a yield of 175 ml with 2% (w/v) xylose concentration.

2.6.3.4 Temperature

Microbial consortia responsible for hydrogen production can be broadly categorised into two groups; mesophiles (30 -40 ^oC) and thermophiles (45-55 ^oC), thus indicating the sensitivity of hydrogen production to variations in environmental temperature (Mohammadi et al., 2012). In addition, a precipitous decrease in temperature has been shown to reduce the hydrogen concentration rapidly; however, this can be improved by the microorganisms adapting to the new temperature conditions (Huang et al., 2004). Studies have reported the effects of temperature on biohydrogen production, ranging from mesophilic (35 ^oC) to extreme thermophilic (>65 ^oC) (Reilly et al., 2014; Kongjan and Angelidaki, 2010). Furthermore, operational temperature has been shown to affect the metabolic pathways, which also influences the composition of the byproducts.

The majority of biohydrogen experiments are conducted under mesophilic conditions for the purpose of economic feasibility and inoculums source (Elshamouby et al., 2013). Thus, most fermentative biohydrogen production processes using lignocellulosic biomass were carried out at mesophilic

temperatures (Moodley and Kana, 2015; Dong et al., 2009; Fan et al., 2006; Cui and Shen, 2012; Fangkum and Reungsang, 2010). Lin et al. (2008) reported a 100% hydrogen production rate improvement by increasing the temperature from 30 ^{o}C to 40 ^{o}C. In another study, maximum glucose degradation (98.1%) was achieved by increasing the temperature from 20 ^{o}C to 40 ^{o}C (Wang and Wan, 2008b). Table 5 shows reported optimum temperatures and hydrogen yields from lignocellulosic materials.

Table 5. Biohydrogen production from different lignocellulosic materials under optimal physico-chemical conditions.

Substrate	**pH**	**Temperature (ºC)**	**HRT**	**Inoculum**	**H_2 yield**	**Reference**
Potato starch	5.25	37	12 hr	*Clostridium butyricum* and *Enterobacter aerogenes* HO-39	2.7 mol mol/ glucose	Yokoi et al., 2002
Rice waste	5.5	37	7 hr	Anaerobic mixed culture	134 mL/g-VS	Dong et al., 2009
Wheat straw	7.0	36	6.25 days	Cow dung compost	68.1 ml/g VS	Fan et al., 2006
Corn stover	6.8	50	40 hr	*Clostridium thermocellum*	250 ml/L/d	Lalaurette et al., 2009
Sugarcane leaves	6.5	37	72 hr	Anaerobic sewage sludge	248ml/g total sugar	Moodley and Kana, 2015
Dry grass	7.0	35	25 hr	*Clostridium pasteurianum*	72.21ml/g dry grass	Cui and Shen, 2012
Cassava	5.5	37	20 hr	Seed sludge from cassava wastewater	186 ml/g COD	Sreethawong et al., 2010
Sugarcane baggase	6.5	37	400hr	Elephant dung	0.84 mol/mol total sugar	Fangkum and Reungsang, 2010

2.5.7 Scale-up and techno-economic analysis of biohydrogen production processes

Dark fermentation experiments in reported literature are frequently conducted in lab-scale reactors, and limited studies have reported scale-up production studies (Ghimire et al., 2015). The scale up of a fermentation process is governed by several important engineering considerations, which often dictate process performance (Hewitt and Noenow, 2010). Furthermore, there is a lack of studies reporting on the optimization of fermentation conditions across scales. Ren et al. (2006) carried out fermentative hydrogen production in a 1.48m^3 capacity reactor and reported a hydrogen fraction ranging from 40 – 52% and a yield of 26.13 mol/ kg COD molasses removed. Another study obtained a yield of 72 ml H_2/ g VS from kitchen waste in a 0.15 m^3 inclined plug-flow reactor (Jayalakshmi et al., 2009). In a scale up study from 500 ml to 8000 ml using a 13L continuous stirred tank reactor, Moodley and Kana (2015) reported a hydrogen yield of 248.05 ml/g fermentable sugar with an HRT of approximately 65 hr.

An economic assessment is the determination of the feasibility of a bioprocess in which the technical aspects are coupled with the economic aspects thus allowing the investment and production costs to be projected (Swanson et al., 2010). Techno-economic analysis provides invaluable insight into the feasibility of scaling a process industrially (Qureshi et al., 2013). Classen et al. (2000) performed a techno-economic analysis with a bioreactor capacity of 95 000 L. The simulated plant produced 39 kg H_2/ hr using organic waste materials. A unit production cost of \$3.65 kg/H_2 was estimated, however, several key cost considerations such as personnel, feedstock and construction costs were not factored into the operating expenses. Li et al. (2012) compared the profitability of two simulated models, both producing hydrogen but using either wastewater or agricultural residues. Revenue from hydrogen sales remained unchanged; however, the 400 m^3 plant using agricultural waste was shown to be 30% more profitable than the 300 m^3 plant using wastewater.

2.6 Conclusion

Sugarcane is an important agricultural crop produced worldwide and the leaf residues are usually burnt prior to harvest, posing significant environmental hazards. These leaves are rich in fermentable sugars that may be recovered using a suitable pretreatment strategy for biofuel production. Fermentative hydrogen is a clean and renewable source of energy. Its production from lignocellulosic biomass is an attractive approach for renewable energy development since these materials are low-cost, abundant and sustainable. Ultimately, a techno-economic analysis of this biofuel from waste sugarcane leaves will provide data for scale-up and commercialization.

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Chapter 3

Assessment of three optimized models for the production of xylose and glucose from waste sugarcane leaves using different acid-based hybrid pretreatments for biohydrogen production

Abstract

Waste sugarcane leaves as agricultural residues are potential feedstocks for biofuel production. This paper reports the development of three optimized models for the production of xylose and glucose from waste sugarcane leaves using acid-based hybrid pretreatment strategies. The input variables for each model consisted of acid concentration, temperature, solid to liquid (S:L) ratio and heating time in the range of 0.5–5.0% (v/v), 60–100 ^{o}C, 30–50% (w/v) and 60–240 min, respectively. The hybrid pretreatments were based on HCl, H_2SO_4 and HNO_3. All models showed coefficients of determination (R^2) above 0.78. Process optimization gave xylose and glucose yields of 78 g/L and 11.48 g/L, 50.75 g/L and 7.15 g/L, 30.82 g/L and 3.99 g/L for HCl, H_2SO_4 and HNO_3 based hybrid pretreatments, respectively. HCl based pretreatment of 4.90% HCl at 99 ^{o}C for 84 min with a S:L of 47.26% showed a high solubilization of hemicellulose (93.15%), with a xylose: glucose ratio of 6.8:1 and a short pretreatment time. The interactive effect of these input parameters on the sugar recovery pattern revealed that increasing acid concentration and heating temperature from 0.5 to 5.0% and 60 ^{o}C to 100 ^{o}C, respectively, resulted in higher yields of glucose and xylose. Preliminary assessment of these fermentable sugars on dark fermentation gave a peak hydrogen fraction of 40.11% and a yield of 18.6 ml H_2 g^{-1} fermentable sugar. Optimized data suggested that a significant yield of these fermentable sugars can be recovered from these wastes. The recovered sugars are excellent substrates for various bioprocesses. These findings highlight alternative methods for managing sugarcane leaf wastes towards biofuel generation.

Keywords: Sugarcane leaves feedstock, lignocellulosic feedstock pretreatment, biohydrogen production, bioprocess optimization

1. Introduction

The global dependency on non-renewable fossil fuels and the current emission of greenhouse gases are propelling research towards a cleaner and more sustainable source of energy (Faloye et al., 2014). A major factor that influences the production cost of biofuels is the feedstock (Serra and Zilberman, 2013). Plant biomass is increasingly being considered as a suitable feedstock due to its low costs, high availability and the added environmental benefits (Lopez-Linares et al., 2013). Annually, 200 billion tons of biomass is produced globally (Zhang, 2008) and the current disposal practice mainly involves burning or landfill dumping (Rashidi et al., 2012).

Sugarcane is an important agricultural crop cultivated worldwide (Jutakanoke et al., 2012). Its annual production amounts to 65 million tons in Thailand (Nguyen et al., 2010), 590 million tons in Brazil (Rocha et al., 2014) and 20 million tons in South Africa (Smithers, 2014). The leaf component of the sugarcane, commonly referred to as trash, constitutes 40% of the sugarcane plant (Nguyen et al., 2010; Smithers, 2014). The leaves are considered waste and are often burnt prior to harvesting (Jutakanoke et al., 2012). Several studies have reported an increase in the emission of harmful mutagenic particulate matter such as benzo(b)fluoranthene and benzo(a)pyrene during harvesting periods (Silva et al., 2010; Cristale et al., 2012; de Andrade et al., 2011). These polycyclic aromatic hydrocarbons have been found to affect the functioning of the lungs as well as other health ailments during exposure (Prado et al., 2012).

Sugarcane leaves are composed of 36% cellulose, 21% hemicellulose and 16% lignin (Eggleston et al., 2014). Lignocellulosic biomass is recalcitrant towards microbial decomposition owing to its rigid and crystalline structure of cellulose, which is enclosed by a cross-linked matrix of hemicellulose and lignin (Kim and Mazza, 2008). Thus, appropriate low cost and efficient pretreatments are essential for the release of the fermentable sugars for industrial bioprocesses. The pretreatment regime is dependent on the type of lignocellulose present since biomass has a high level of variability in complexity (Zheng et al., 2014). Current pretreatment strategies include physical (milling, extrusion, microwave), chemical (alkali, acid, ionic liquid) and physico-chemical (steam, ammonia fiber explosion) (Mood et al., 2013).

Acid pretreatment is a widely used technique since it is inexpensive and is effective in solubilizing hemicellulose into its monomeric sugars while reducing cellulose crystallinity (Donghai et al., 2006). Significant strides have been made towards optimizing sugar release following acidic pretreatment on a variety of lignocellulosic material (Lopez-Linares et al., 2013; Gil et al., 2010; Dagnino et al., 2013; Kamireddy et al., 2013). The pretreatment of sugarcane leaves is scarcely reported (Jutakanoke et al., 2012; Moutta et al., 2012). In addition, there is a lack of substantial detailed studies modeling the

interaction of pretreatment input parameters on both the release pattern of xylose and glucose from lignocellulosic biomass.

Response surface methodology (RSM) has been widely employed for the modeling and optimization of bioprocesses (Wu et al., 2015). Various studies have modeled the release of fermentable sugars using RSM with high efficiency (Dagnino et al., 2013; Kim and Mazza, 2008; Moodley and Kana, 2015; Du et al., 2013).

This study aims at comparing the efficiency of three acid-based hybrid models to optimize the release of fermentable sugars from sugarcane leaves with a focus on the influence of main operational parameters such as acid concentration, heating temperature, heating time and solid: liquid ratio. Additionally, the use of these fermentable sugars in dark fermentation for hydrogen production is assessed.

2. Materials and methods

2.1 Raw material

The sugarcane leaves used in this study were collected at 8 months old from The South African Sugarcane Research Institute (SASRI) located on the North Coast of South Africa (29° 42' 18" S, 31° 02' 44" E) at an altitude of 96 m. This area is characterized by a warm climate with an annual mean rainfall of 951 mm. The leaves, cut roughly at the third to sixth leaf, were transported in sealed plastic bags, then dried at 60 ^{o}C for 72 hours followed by milling using a centrifugal miller (Retsch ZM-1, Durban, South Africa) with a 1 mm sized mesh to yield particles sized ≤ 1 mm. Milled leaves were stored in sealed paper bags prior to use.

2.2 Experimental design

The optimization window for the input pretreatment parameters was selected with the view to minimize the energy input, while enhancing xylose and glucose recovery and guided by previous reports (Moutta et al., 2012; Zhang et al., 2011; Gil et al., 2010; Moodley and Kana, 2015). The input parameters consisted of acid concentration, temperature, solid to liquid (S:L) ratio and heating time in the range of 0.5–5.0% (v/v), 60–100 ^{o}C, 30–50% (w/v) and 60–240 min, respectively. Three acid types used were HCl, H_2SO_4, and HNO_3. The Box-Behnken design was used to generate 29 experiments with varied pretreatment input conditions for each of the acid-based hybrid pretreatment models (Table 1), thus, a total of 87 experiments were carried out in duplicate, and consequently, a total of 174 experiments were evaluated.

Table 1. Coded and actual levels of the input variables for the experimental design.

Independent variables	Symbols	Coded		
		-1	0	1
Acid concentration (%, v/v)	A	0.5	2.75	5.0
Solid: Liquid ratio (%, w/v)	B	30	40	50
Heating temperature (°C)	C	60	80	100
Heating time (min)	D	60	150	240

2.3 Pretreatment process

Specific amounts of milled sugarcane leaves (6.0, 8.0, 10.0 g) were transferred into 250 ml Schott bottles and 20 ml of varied concentrations of acid (0.5, 2.75, 5.0% (v/v)) was then added. The contents were mixed and heated using a PolyScience Analogue water bath. The solid to liquid ratio (S: L), acid concentration, heating time and heating temperature setpoints were maintained as specified in the design (Tables 2–4). Timing was initiated once the temperature of the substrate reached the specified setpoint. The pretreated sugarcane leaves were filtered and the solid fraction was washed three times with distilled water for chemical and morphological examination. The liquid fraction was analyzed for the concentration of glucose and xylose.

2.4 Scanning electron microscopic analysis

Physical changes in native and pretreated sugarcane leaves were analyzed by scanning electron microscopy (ZEISS EVO LS 15). All samples were mounted on conductive adhesive tape, sputter coated with gold (Eiko IB-3 Ion Coater) and observed at 5 kV voltages. Images were taken at 277x and 350x magnification.

2.5 Preliminary assessment for hydrogen production

2.5.1 Seed inoculum

The anaerobic sludge used in this study was obtained from The Darville Wastewater treatment plant (Pietermaritzburg, South Africa). Heat treatment was applied to the sludge (121 ^{o}C for 10 minutes) to reduce methanogenic activity while preserving the hydrogen producing, spore-forming microorganisms.

2.5.2 Experimental set-up and dark fermentation process

A modified 2L Erlenmeyer flask was used as the reactor vessel. The reactor was fed with 300 ml of treated anaerobic sludge, 600 ml of the optimally pretreated sugarcane leaves and supplemented with 600 ml of inorganic salts (g/l): NH_4CL 0.5, KH_2PO_4 0.5, K_2HPO_4 0.5, $NaHCO_3$ 4.0, $FeCl_3$ 0.15, $MgCl.6H_20$ 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, $Na_2MoO_2.2H_2O$ 0.03. The final sugar concentration was 9.24 ± 0.14 g/L and 1.41 ± 0.21 g/L for xylose and glucose, respectively. The pH was adjusted to 6.5 using 1M NaOH and the flask was flushed with nitrogen gas for 5 minutes to promote anoxic conditions. The fermentation process was carried out in a shaking water bath with operational setpoints of 180 rpm, 37 ^{o}C and 72 hours for agitation, temperature and HRT, respectively.

2.6 Analytical methods

Xylose and glucose released from each experimental run were quantified using the Biochemistry Analyzer (Model 2700 select-dual configuration, YSI, USA), which uses the principle of enzyme coupled reactions and electrochemical detection. Experimental data were analyzed using the statistical software Design-Expert, Stat-Ease Inc., USA. The fiber composition of native and pretreated leaf samples was analyzed using detergent fiber analysis techniques as described by Goering and Van Soest (1970). Cellulose, hemicellulose and lignin composition were calculated according to Wolfrum et al. (2009). The evolution of the biogas from the fermentation process was monitored in real time using three sensors namely hydrogen (BCP-H_2), methane (BCP-CH_4) and carbon dioxide (BCP-CO_2) (Bluesens, Germany) sampling every minute. The quantification of the biogas volume was achieved using a milligas counter (MGC, Bluesens, Germany).

3. Results and discussion

3.1 Development of pretreatment models and optimization

Four independent input variables viz. acid concentration (A) (%, v/v), solid to liquid ratio (B) (%, w/v) heating temperature (C) (^{o}C) and heating time (D) (min) were used in the design matrix with glucose and xylose recovery representing the model responses (Table 1). The pretreatment responses for HCl, H_2SO_4 and HNO_3 are shown in Tables 2, 3 and 4, respectively. The experimental data were used to calculate the coefficients of the independent variables of the quadratic Equations (1,2), (3,4) and (5,6) for the HCl model, H_2SO_4 model, and HNO_3 model, respectively.

Table 2. Xylose and glucose released from HCl based hybrid pretreatments.

Run	A: HCl (%)	B: Heating time (mins)	C: Heating temperature (^{0}C)	Solid: Liquid %	Response 1 Xylose (g/L)	Response 2 Glucose (g/L)
1	2.75	240	80	50	57.82	8.94
2	0.50	60	80	40	0.79	3.99
3	2.75	240	60	40	5.83	4.66
4	0.50	240	80	40	0.43	3.29
5	0.50	150	80	50	0.39	2.46
6	5.00	150	60	40	5.83	3.46
7	2.75	150	100	30	50.96	10.10
8	2.75	150	80	40	0.29	3.42
9	2.75	60	80	50	34.58	7.89
10	5.00	150	100	40	62.13	14.57
11	2.75	60	80	30	8.38	5.24
12	0.50	150	100	40	0.50	3.42
13	2.75	150	80	40	44.04	7.98
14	5.00	240	80	40	46.09	9.30
15	2.75	60	100	40	57.61	9.25
16	2.75	240	80	30	31.94	4.96
17	5.00	150	80	30	39.57	6.73
18	5.00	60	80	40	54.83	8.11
19	5.00	150	80	50	63.38	10.99
20	0.50	150	80	30	0.43	4.13
21	2.75	150	80	40	37.32	5.15

22	2.75	240	100	40	37.37	7.67
23	2.75	150	80	40	23.81	6.14
24	2.75	150	60	50	0.51	3.98
25	2.75	150	60	30	3.30	4.27
26	2.75	150	80	40	47.71	7.43
27	2.75	150	100	50	30.80	6.79
28	2.75	60	60	40	1.74	5.55
29	0.50	150	60	40	0.45	3.67

Table 3. Xylose and glucose released from H_2SO_4 based hybrid pretreatments.

Run	A: H_2SO_4 (%)	B: Heating time (mins)	C: Heating temperature (^{0}C)	Solid: Liquid %	Response 1 Xylose (g/L)	Response 2 Glucose (g/L)
1	2.75	150	100	50	33.7	4.65
2	2.75	240	80	30	23.91	3.51
3	2.75	240	60	40	1.13	2.59
4	2.75	150	80	40	18.59	4.08
5	0.5	150	60	40	0.47	3.59
6	2.75	60	60	40	0.66	3.57
7	2.75	60	80	30	4.92	3.11
8	2.75	150	60	30	0.79	2.70
9	0.5	150	100	40	0.78	6.18
10	2.75	240	100	40	30.86	6.13
11	5.0	150	80	30	37.30	6.68
12	0.5	150	80	30	0.45	3.37
13	2.75	150	60	50	1.22	6.99
14	0.5	150	80	50	0.51	4.91
15	0.5	240	80	40	0.39	3.76
16	2.75	240	80	50	17.90	6.26
17	5.0	150	80	50	35.15	7.23
18	2.75	60	80	50	5.85	7.33
19	2.75	150	80	40	13.44	4.91
20	2.75	150	80	40	15.74	5.55
21	2.75	150	80	40	24.89	7.88

22	5.0	60	80	40	23.70	6.02
23	0.5	60	80	40	0.58	5.13
24	2.75	150	80	40	15.99	6.62
25	5.0	150	100	40	47.75	9.23
26	5.0	150	60	40	3.67	5.46
27	2.75	150	100	30	37.19	5.96
28	2.75	60	100	40	33.96	5.70
29	5.0	240	80	40	44.32	8.48

Table 4. Xylose and glucose released from HNO_3 based hybrid pretreatments.

Run	A: HNO_3 (%)	B: Heating time (mins)	C: Heating temperature (^{0}C)	Solid: Liquid %	Response 1 Xylose (g/L)	Response 2 Glucose (g/L)
1	5.0	150	80	50	5.49	4.41
2	5.0	150	60	40	1.59	3.84
3	2.75	240	80	50	11.90	4.12
4	5.0	60	80	40	3.85	3.42
5	5.0	240	80	40	7.06	3.51
6	0.50	150	80	30	0.08	1.21
7	0.50	150	100	40	0.11	2.71
8	2.75	240	80	30	12.25	2.27
9	5.00	150	80	30	12.58	2.95
10	2.75	60	80	50	3.03	4.16
11	2.75	150	80	40	11.25	3.96
12	2.75	60	80	30	3.83	2.60
13	0.50	60	80	40	0.13	1.63
14	2.75	150	80	40	10.63	4.16
15	0.50	150	60	40	0.21	1.28
16	2.75	150	80	40	10.58	3.34
17	2.75	240	100	40	44.06	6.03
18	0.50	150	80	50	0.23	2.29
19	0.50	240	80	40	0.09	1.59
20	2.75	150	80	40	8.92	3.80
21	2.75	150	60	30	0.28	2.59

22	2.75	240	60	40	0.59	3.76
23	2.75	150	100	50	39.80	5.03
24	2.75	60	100	40	32.40	4.50
25	2.75	150	60	50	0.58	4.18
26	2.75	150	80	40	9.25	4.28
27	5.0	150	100	40	16.35	2.78
28	2.75	150	100	30	41.93	6.80
29	2.75	60	60	40	0.24	3.51

HCl based model

$$\text{Xylose (g/L)} = 6.02 + 2.68A + 0.47B + 2.18C - 0.10D + 1.48AB + 2.84AC + 0.47AD - 0.75BC + 0.33BD - 0.17CD - 0.14A^2 + 0.16B^2 + 0.27C^2 + 0.45D^2 \quad (1)$$

$$\text{Glucose (g/L)} = 30.64 + 22.40A + 4.41B + 18.48C + 1.80D + 5.97AB + 14.06AC - 2.09AD - 4.34BC - 0.083BD - 6.08CD - 5.78A^2 + 0.12B^2 - 8.01C^2 + 2.04D^2 \quad (2)$$

H_2SO_4 based model

$$\text{Xylose (g/L)} = 17.73 + 15.73A - 0.85B + 14.69C + 4.07D - 0.55AB + 10.94AC + 5.20AD - 0.98BC - 1.74BD - 0.89CD - 0.61A^2 - 0.13B^2 - 0.97C^2 - 1.47D^2 \quad (3)$$

$$\text{Glucose (g/L)} = 5.81 + 1.35A + 1.00B + 1.08C - 0.011D - 0.25AB + 0.29AC + 0.96AD - 1.40BC - 0.37BD + 0.35CD + 0.49A^2 - 0.42B^2 - 0.42C^2 - 0.56D^2 \quad (4)$$

HNO_3 based model

$$\text{Xylose (g/L)} = 10.13 + 3.84A - 0.83B + 14.26C + 2.71D - 1.81AB + 3.71AC + 0.81AD - 0.61BC + 0.11BD + 2.83CD - 9.04A^2 + 1.49B^2 + 7.26C^2 - 0.079D^2 \quad (5)$$

$$\text{Glucose (g/L)} = 3.91 + 0.85A + 0.48B + 0.72C + 0.12D + 0.097AB - 0.62AC + 0.033AD - 0.84BC + 0.073BD + 0.32CD - 1.39A^2 - 9.342E\text{-}003B^2 + 0.54C^2 - 0.20D^2 \quad (6)$$

These model equations illustrate the influence of each input factor and the interactive effect of the factors on the response of xylose and glucose yield. The significance of these models was assessed using analysis of variance (Tables 5 and 6). The coefficients of determination (R^2) of 0.80, 0.93 and 0.81 were obtained for HCl, H_2SO_4 and HNO_3 based pretreatments respectively for the xylose models. Thus, these models could account for 80%, 93% and 81% of variations in the observed data, respectively. The significance of the models was further established by the F values of 3.90, 13.36 and 4.38 for HCl, H_2SO_4 and HNO_3 pretreatments, respectively. Models for glucose yields from the HCl, H_2SO_4 and HNO_3 based pretreatment showed coefficients of determination (R^2) of 0.86, 0.78 and 0.83, respectively. A perusal of the F values (6.25, 3.64, and 4.94 for HCl, H_2SO_4 and HNO_3 models, respectively) further underscores the significance of these models.

Table 5. Analysis of variance (ANOVA) of quadratic models for optimization of xylose yield.

Source	Sum of squares	df	Mean squares	F-value	P-value	R^2
HCl Model	12248.62	14	874.90	3.90	0.0078	0.80
H_2SO_4 Model	6390.99	14	456.50	13.36	<0.0001	0.93
HNO_3 Model	3911.21	14	279.37	4.38	0.0046	0.81

df: degrees of freedom; F-value: Fisher-Snedecor distribution value; P-value: probability value; R^2: coefficient of determination

Table 6. Analysis of variance (ANOVA) of quadratic models for optimization of glucose yield.

Source	Sum of squares	df	Mean squares	F-value	P-value	R^2
HCl Model	193.11	14	13.79	6.25	0.0008	0.86
H_2SO_4 Model	67.48	14	4.82	3.64	0.0108	0.78
HNO_3 Model	39.68	14	2.83	4.94	0.0025	0.83

df: degrees of freedom; F-value: Fisher-Snedecor distribution value; P-value: probability value; R^2: coefficient of determination

The model equations above were solved using the method of Myers and Montgomery (1995) to determine the optimum setpoints of acid concentration, heating temperature, heating time and S: L ratio for maximum release of xylose and glucose for each acid based hybrid model. The predicted optimum pretreatment setpoints for these models are shown in Table 7 with predicted xylose and glucose yields of 81.70 and 14.67 g/L, 61.33 and 9.25 g/L and 41.93 and 5.64 g/L for the HCl model, H_2SO_4 model and HNO_3 model, respectively. Experimental validation of these predictions done in duplicate gave xylose and glucose yields of 78 ± 2.96 and 11.48 ± 0.45 g/L, 50.75 ± 1.06 and 7.15 ± 0.14g and 30.82 ± 0.63g/L and 3.99 ± 0.20g/L for the HCl model, H_2SO_4 model and HNO_3 model, respectively. The HCl model was more accurate compared to the other investigated models with the observed xylose and glucose being only 3.7 and 3.19 g/L lower than the predicted yield. The observed xylose yields showed a 3.7%, 17.7% and 26.5% discrepancy from predicted values for HCl, H_2SO_4 and HNO_3 models, respectively. Similar discrepancies were observed for glucose yields with observed values lower than predicted values (21.7%, 22.7%, 29.3% for HCl model, H_2SO_4 model and HNO_3 model, respectively). The ratio of xylose to glucose released was 6.8:1, 7:1 and 7.7:1 for HCl model, H_2SO_4 model and HNO_3 model, respectively, indicating xylose release was approximately seven times greater than glucose.

Table 7. Optimum levels of variables during pretreatment of sugarcane leaves.

Independent variables	**Predicted optimum levels**		
	HCl	**H_2SO_4**	**HNO_3**
Acid concentration (%)	4.90	4.84	3.35
Heating time (min)	84.14	210.54	239
Temperature (^oC)	99	98	100
S:L (%)	47.26	36.42	30

	Response (g/L)	**Predicted value**	**Observed value***
HCl model	Xylose	81.70	78 ± 2.96
	Glucose	14.67	11.48 ± 0.45
H_2SO_4 model	Xylose	61.33	50.75 ± 1.06
	Glucose	9.25	7.15 ± 0.14
HNO_3 model	Xylose	41.93	30.82 ± 0.63
	Glucose	5.64	3.99 ± 0.20

*Values depicted are mean ± SD for n = 2

The optimization study showed that substantially more sugar was released compared to previous reports. The pretreatment of sugarcane leaves in a previous study (Moutta et al., 2012) yielded 56.6 g/L under pretreatment conditions of 2.9% H_2SO_4 at 130 ^oC. Glucose recovery from 2.75% H_2SO_4 pretreated Erica was comparably lower (1.6 g/L) at 80 ^oC for 75 min (Gil et al., 2010).

3.2 Xylose and glucose release pattern with HCl based pretreatment

With HCl based hybrid pretreatment (Table 2), a xylose concentration of 63.38 g/L was achieved with 5.0% HCl (v/v), 50% S:L (%, w/v), 80 ^{o}C at 150 min (batch 19) and a glucose concentration of 14.57 g/L was observed with a pretreatment of 5.0% HCl (v/v), 40% S:L (%, w/v), 100 ^{o}C at 150 min (Run 10). Both batches showed that the interaction of HCl concentration and temperature at high setpoint values within the selected search window enhanced the recovery of xylose and glucose. Production of fermentable sugars from lignocellulosic biomass of 22.1 g glucose kg^{-1} wheat straw with 1% HCl pretreatment (Tutt et al., 2012) and of 29.4 g/L from 1.2% HCl baggase pretreatment (Wang et al., 2015) have been reported. The interactive effect of pretreatment input parameters on glucose yield are illustrated in the response surface graphs (Figs. 1A) showing that an increase in temperature and HCl concentration from 60 ^{o}C to 100 ^{o}C and 0.5% to 5.0%, respectively, resulted in an increase in glucose from 3.5 g/L to 14 g/L. A similar trend was observed for xylose (Figs. 1C) where the yield increased from 5 g/L to 72 g/L when HCl concentration and temperature were increased concurrently from 0.5% to 5.0% and 60 ^{o}C to 100 ^{o}C. De Vasconcelos et al. (2013) observed a similar trend with the increase in temperature and acid concentration setpoints enhancing the conversion of cellulose to glucose from 10% to 60%. By contrast, the interactive effect of time and HCl concentration (Fig. 1B) and temperature and S:L (Fig. 1D) exerted very little influence on the yield of glucose and xylose respectively.

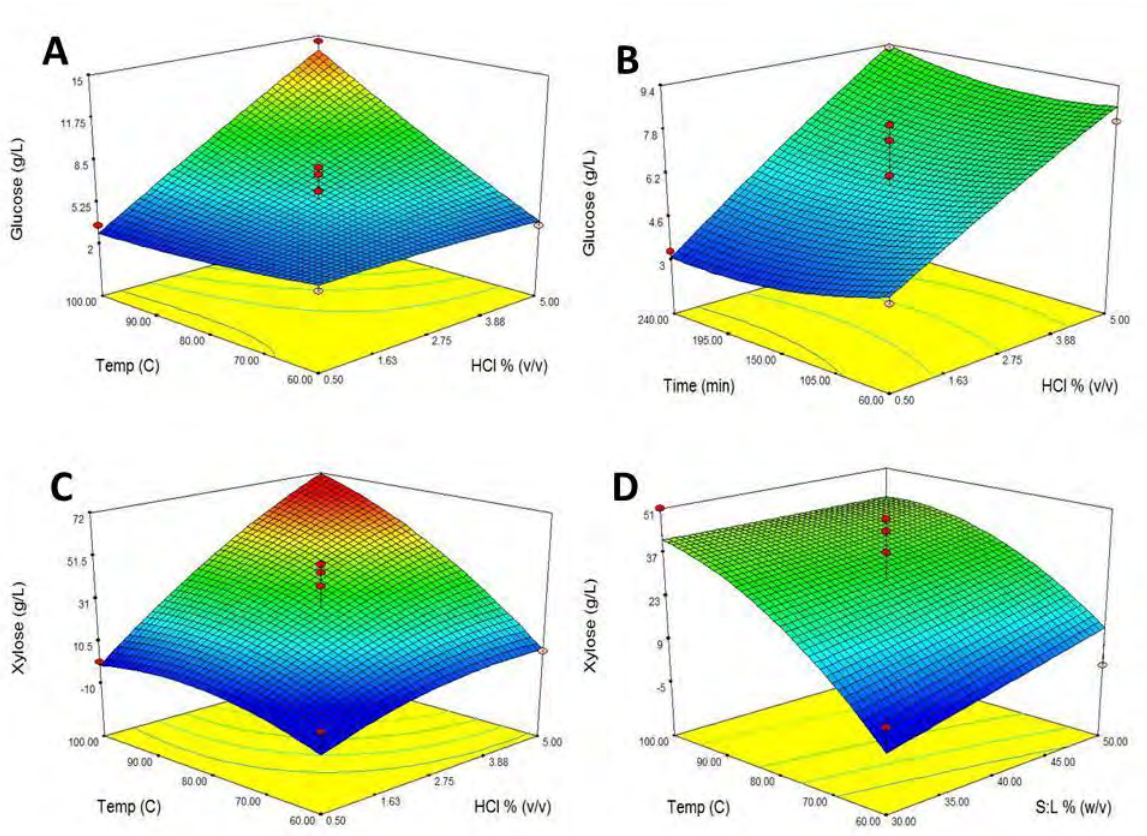

Fig. 1. Response surface plots for HCl pretreatment showing the interactive effect between: (A) temperature and HCl concentration; (B) heating time and HCl concentration; (C) temperature and HCl concentration; and (D) temperature and solid: liquid ratio on the yield of sugar.

3.3 Xylose and glucose release pattern with H_2SO_4 based pretreatment

Using the H_2SO_4 based hybrid pretreatment (Table 3), xylose and glucose concentrations of 47.75 g/L and 9.23 g/L, respectively, were obtained using 5.0% H_2SO_4 (v/v), 40% S:L (%, w/v) at 100 ^oC for 150 min (batch 25). Thus, higher yields of xylose and glucose are obtained at a higher concentration of H_2SO_4 and temperature setpoint values compared to low yields of these sugars (0.39 g/L and 3.76 g/L for xylose and glucose, respectively) using 0.5% H_2SO_4 (v/v), 40% S: L (%, w/v) at 80 ^oC for 240 min (batch 15). Moutta et al. (2012) reported a xylose recovery of 4.98 g/L from sugarcane leaves using 0.5% H_2SO_4 (w/v) at 110 ^oC. On the contrary, a 10-fold increase in xylose yield was observed in the present study using a higher acid concentration (5.0% H_2SO_4) coupled with a lower temperature (100 ^oC). The interactive effect of the input parameters on the yield of fermentable sugar is shown in Fig. 2, with a gradual increase of glucose recovery up to 9 g/L when the temperature and H_2SO_4 concentration were increased from 60 to 100 ^oC and 0.5% to 5.0%, respectively (Fig. 2A). These findings are in line with Xu et al. (2011), where a simultaneous increase in temperature and acid concentration from 140 to 160 ^oC and 0.50 to 1.10% yields an increase in glucose recovery from 20 to 80% from sorghum leaves. A noticeable increase in xylose concentration from 0.4 to 58 g/L was observed when the temperature and acid concentration was increased from 60 ^oC to 100 ^oC and 0.5% to 5.0%, respectively (Fig. 2C). The effect of heating time on sugar recovery was shown to be negligible as an increase in heating time from 60 min to 240 min resulted in low increments in sugar yield, 0 g/L and 0.6 g/L for xylose (Fig. 2D) and glucose (Fig. 2B), respectively.

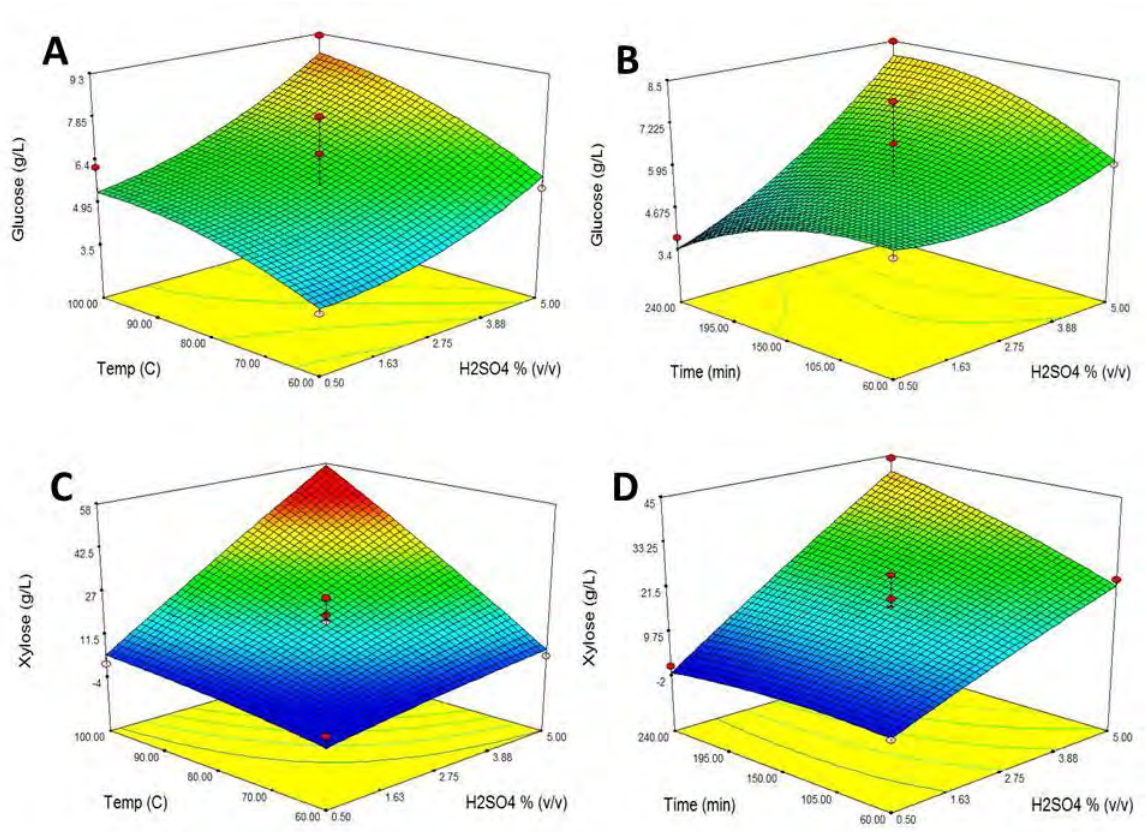

Fig. 2. Response surface plots for H_2SO_4 pretreatment showing the interactive effect between: (A) temperature and H_2SO_4 concentration; (B) heating time and H_2SO_4 concentration; (C) temperature and H_2SO_4 concentration; and (D) heating time and H_2SO_4 concentration on the yield of sugar.

3.4 Xylose and glucose release pattern with HNO_3 based pretreatment

Sugar recovery after pretreatment with HNO_3 is shown in Table 4. A xylose concentration of 44.07 g/L was achieved using 2.75% HNO_3 (v/v), 40% S: L (%, w/v) at 100 ^oC for 240 min (Run 17). A glucose recovery of 6.81 g/L was obtained using 2.75% HNO_3 (v/v), 30% S: L (%, w/v) at 100 ^oC for 150 min (Run 28). High yields of xylose (19.7 g/100 g) have been reported from the hydrolysis of corn stover at 100 ^oC with 0.2% HNO_3 (Zhang et al., 2011). The interactive effect of the input parameters on the yield of xylose and glucose showed that an increase in HNO_3 concentration from 0.5% to 3.88% while maintaining temperature at 60 ^oC resulted in an increase in glucose from 0.8 g/L to 3.5 g/L (Fig. 3A). The yield of xylose increased significantly from 0 to 33 g/L when HNO_3 concentration was kept constant at 2.75% while simultaneously increasing temperature from 60 to 100 ^oC (Fig. 3C), indicating that temperature has a greater interactive effect on the yield of xylose. Kim et al. (2014) investigated the pretreatment of rice straw and reported an increase in xylose recovery from 0 to 56 g/L when HNO_3 was kept constant at 0.40% and temperature was increased from 140 to 180 ^oC.

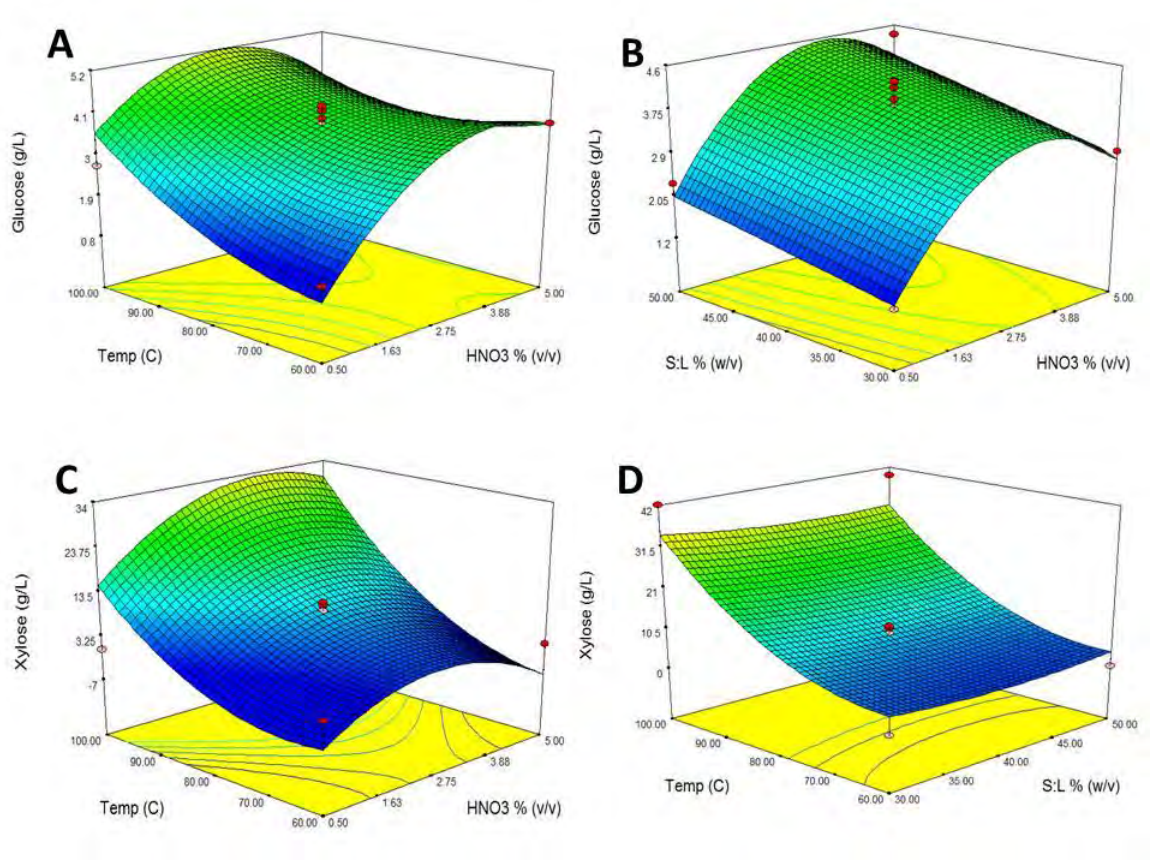

Fig. 3. Response surface plots for HNO_3 pretreatment showing the interactive effect between: (A) temperature and HNO_3 concentration; (B) solid: liquid ratio and HNO_3 concentration; (C) temperature and HNO_3 concentration; and (D) temperature and solid: liquid ratio on the yield of sugar.

3.5 Comparative assessment of the three hybrid pretreatments

The yields of xylose and glucose from the three hybrid pretreatments showed high variability (3.99–11.48 g/L and 30.82–78 g/L, respectively), thus underscoring the sensitivity of xylose and glucose recovery with the considered input parameters. Under optimum conditions xylose yields of 78, 50.75 and 30.82 g/L was recovered with HCl, H_2SO_4 and HNO_3 pretreatment, respectively. The high yield of xylose with HCl based pretreatment is attributed to the ability of HCl to permeate lignocellulosic material more easily compared to other acids (Demirbas, 2008). A similar rationale may explain the high yield of glucose (11.48 g/L) obtained under HCl pretreatment conditions. Maximum hemicellulose solubilization (93.15%) was observed with HCl while 92% was achieved with H_2SO_4; however, the yield of xylose was 27.25g/L lower with H_2SO_4. Similarly, nitric acid showed a solubilization of 79% but exhibited a significantly lower yield in xylose by 47.18 g/L compared to HCl. A consequence of acidic pretreatment is the loss of some sugars through degradation in the hydrolysate (Castro et al., 2011). This would elucidate the high dissolution of hemicellulose and subsequent low yields of xylose. Five-carbon sugars such as xylose degrade more rapidly compared to other sugars (Bensah and Mensah, 2013). The xylose: glucose ratios of 6.8:1, 7:1 and 7.7:1 were obtained for the HCl model, H_2SO_4 model and HNO_3 model, respectively. Thus, a slightly higher ratio of xylose: glucose could be achieved with HNO_3 pretreatment compared to HCl and H_2SO_4. A comparable ratio of 6.1:1 was observed using HCl based pretreatment of Napier grass (Mafuleka and Gueguim Kana, 2015), while a ratio of 5.81:1 was reported with H_2SO_4 pretreatment of grain (Xu and Hanna, 2010). Among the acids examined, HCl hydrolyzed sugarcane leaves released maximum xylose and glucose while subsequently requiring the lowest treatment time of 84 min compared to H_2SO_4 and HNO_3 which require treatment time beyond 210 min, thus signifying the effectiveness of HCl pretreatment.

In an effort to reduce pretreatment cost, the current pretreatment technique does not require a subsequent enzymatic process, which is often used to enhance the yield of fermentable sugar (Mood et al., 2013). Circumventing this additional step will significantly reduce process cost and mitigate the cost associated with the analysis of enzymatic inhibitors formed during acidic pretreatment.

3.6 Biomass analysis

The structural composition of the native and pretreated sugarcane leaves is presented in Table 8 with 40.45% cellulose, 33.14% hemicellulose and 5.85% lignin content in the native sample, which are in close range to the reported values of 36% cellulose, 21% hemicellulose and 16% lignin (Eggleston et al., 2014). Variation in structural composition can occur depending on the growing location, harvesting period and analytical procedure (Du et al., 2013). The acidic pretreatments significantly solubilized the hemicellulose structure with 93.15%, 92.46% and 79.36% for HCl, H_2SO_4 and HNO_3 pretreatment, respectively (Table 8). This solubilization of hemicellulose accounts for the relatively high yields of xylose recovery obtained. Surprisingly, the cellulose and lignin content was shown to increase after acidic pretreatment. Samuel et al. (2011) reported similar observations where acidic pretreatment of switchgrass increased cellulose and lignin content by 8.8% and 21.7%, respectively, while decreasing the hemicellulose content 18.9%. Similarly, the cellulose content of Miscanthus, sida and sorghum increased by 3.8%, 6.7% and 7.1%, respectively, with acid based pretreatment (Michalska et al., 2012). It is postulated that the increase in lignin content is attributed to the depolymerization and subsequent repolymerization that occur during pretreatment, and this accounts for the substantial accumulation of acid insoluble material (Li et al., 2007). Acidic pretreatment increases cellulose crystallinity, which causes an observed increase in cellulose content (Sun et al., 2014). Moreover, the effect of pretreatment on lignin, cellulose and hemicellulose content differs significantly due to differences in the chemical composition of different biomass residues (Singh et al., 2015).

Table 8. Chemical composition of native and pretreated sugarcane leaves.

Sample	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Native	40.45	33.14	5.85
HCl treated	55.16	2.27	13.61
H_2SO_4 treated	57.19	2.5	14.93
HNO_3 treated	60.41	6.84	9.35

The scanning electron micrographs revealed that acidic pretreatment caused major morphological changes to the sugarcane leaf (Fig. 4). Untreated sugarcane leaves exhibited an intact outer surface (Fig. 4A) compared to the visible physical damage with pretreated samples (Figs. 4B, C, D). Similar observations of pretreated bamboo were reported after aqueous ammonia and dilute acid pretreatment (Xin et al., 2015).

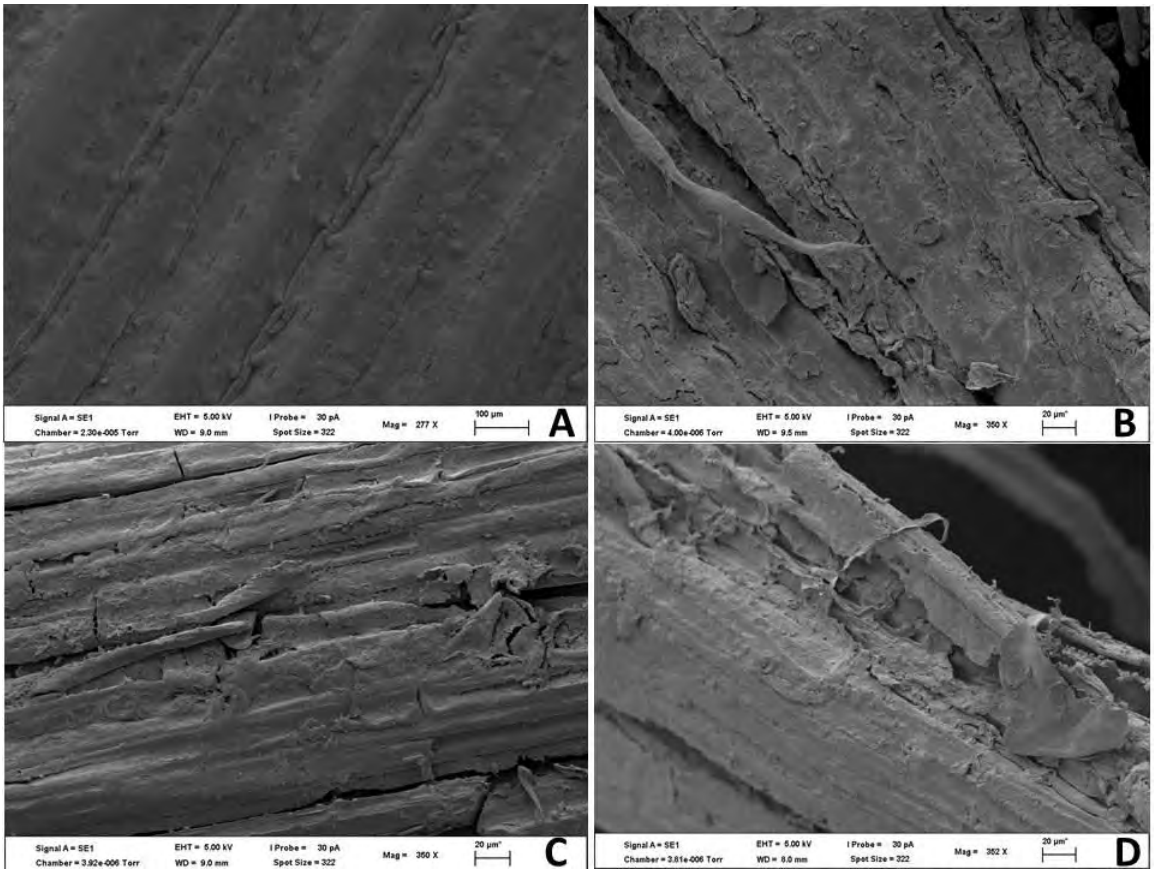

Fig. 4. Scanning electron micrographs showing: (A) native sugarcane leaf; (B) optimally pretreated sugarcane leaf with HCl; (C) optimally pretreated sugarcane leaf with H_2SO_4; (D) optimally pretreated sugarcane leaf with HNO_3.

3.8 Biohydrogen production assessment from optimally pretreated sugarcane leaves

Hydrogen production from pretreated sugarcane leaves showed an initial lag phase of 15 hours (Fig. 5A), which coincided with the depletion of glucose in the medium from 1.41 g/L to 0.15 g/L. As previously observed in a similar process (Moodley and Kana, 2015), there is a preferrential affinity for glucose and the cells initiate xylose metabolism only when the concentration of glucose is near 0%. A similar lag phase of 16 hours on xylose substrate was reported by Cheng et al. (2012). The exponential phase lasted 37 hours with a peak hydrogen fraction of 40.11% (Fig. 5A) and a cumulative volume of 191.53 ml (Fig. 5B) corresponding to a yield of 18.6 ml H_2 g^{-1} fermentable sugar. Hydrogen is produced during the exponential growth phase via the acidogenic pathway in Clostridia. According to Sekoai and Gueguim Kana (2014), during this acidogenic process, *Clostridium* species hydrolyze the substrate either via the acetate or butyrate fermentation pathways to produce hydrogen. An exponential production of carbon dioxide was observed between the 21st and 25th hour of fermentation with a peak fraction of 34.38% and cumulative volume of 169.33 ml (Fig. 5). The relatively high CO_2 is most likely a result of the acetic acid pathway being favoured where 3 mol CO_2 mol^{-1} xylose is formed (Temudo et al., 2009). The final sugar concentration in the effluent was 0.06 ± 0.08 g/L and 0.15 ± 0.005 g/L for xylose and glucose, respectively, indicating 99.3% and 80.1% of xylose and glucose were consumed, respectively.

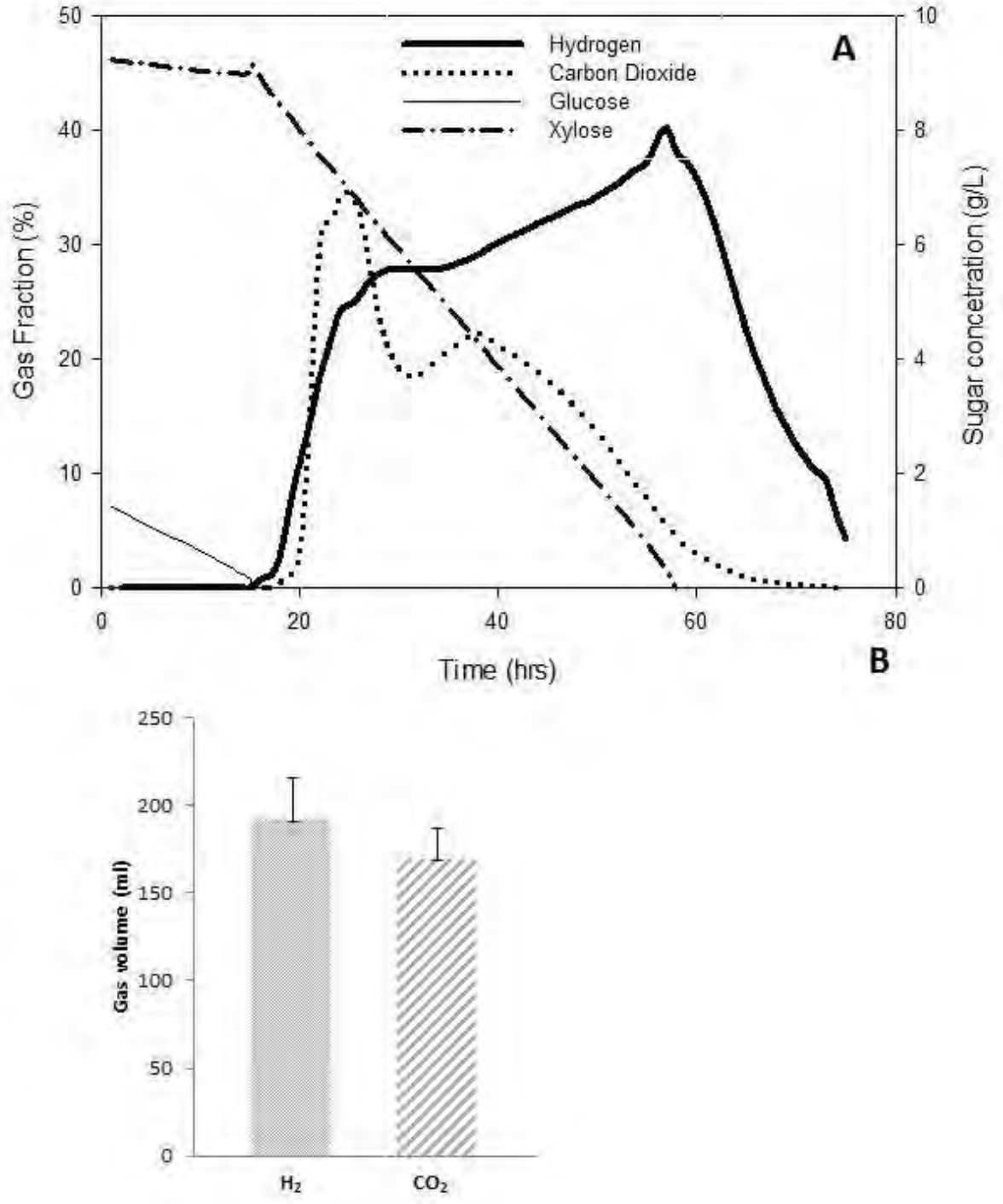

Fig. 5. Biogas production using pretreated sugarcane leaves (A) peak biogas fractions and (B) cumulative biogas volumes.

4. Conclusion

Among the various pretreatment models examined, the HCl model was the most effective since a 93.15% solubilization of hemicellulose was achieved using 4.90% HCl at 99 ^{o}C for 84 min with a S:L of 47.26%. Pretreatment with HCl required the shortest heating time, thus reducing the energy input compared to H_2SO_4 and HNO_3, which require heating times greater than 210 min. The recovered xylose and glucose was subsequently directed for hydrogen production. A peak hydrogen fraction of 40.11% coupled with a yield of 18.6 ml H_2 g^{-1} fermentable sugar was observed. Hydrogen yield can be enhanced by optimizing key process parameters. This study highlighted the viability of using waste sugarcane leaves as a feedstock for the production of xylose and glucose as substrates for biofuel generation.

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Chapter 4

The optimization of physico-chemical parameters for biohydrogen production from waste sugarcane leaves via dark fermentation: A semi-pilot scale assessment

Abstract

This study models and optimizes the operational parameters for biohydrogen production from wastes sugarcane leaves, and assesses hydrogen production on a semi-pilot scale. A Box-Behnken design with input variables of substrate concentration (8–24 g/L), inoculum concentration (10–50% v/v), and hydraulic retention time (HRT, 24–96 hr) was used. A coefficient of determination (R^2) of 0.90 and the predicted optimum operational setpoints of 14.23 g/L substrate concentration, 32.68% inoculum concentration, and 62.77 hr HRT were obtained. Experimental validation produced a biohydrogen yield of 12.76 ml H_2/g fermentable sugar (FS). A semi-pilot scale process in a 13 L Infors reactor under optimized conditions gave a cumulative hydrogen volume and yield of 3739.95 ml and 321 ml H_2 g^{-1} FS respectively, with a peak hydrogen fraction of 37%. Microbial analysis from the process effluent indicated the presence of hydrogen-producing bacteria belonging to *Clostridium* sp., *Klebsiella* sp., and *Enterobacter* sp. These findings illustrate that biohydrogen production from sugarcane leaves can be enhanced under optimal operational conditions. Furthermore, it highlights the scalability of this bioprocess on a semi-pilot scale and provides early stage knowledge for scale-up processes.

Keywords: Biohydrogen production, bioprocess optimization, sugarcane leaves feedstock, semi-pilot scale up, dark fermentation

1. Introduction

The growing global population has increased energy demand, and reserves are becoming exhausted. Fossil fuel oil, coal, and gas are estimated to completely deplete in the next 35, 107, and 37 years respectively [1]. Moreover, concerns have arisen over climate change from fossil fuel combustion. Thus, alternative energies are being pursued [2]. Among the various alternatives, hydrogen (H_2) has been identified as the most favorable owing to its high energy content (122 kJ/g) and relatively clean combustion profile [3,4].

Presently, a large fraction of hydrogen is produced from natural gas [3]. The production of hydrogen via biological pathways such as dark fermentation, photofermentation, and microbial electrolysis cells has shown immense potential. Dark fermentation is considered a promising technology due to its flexibility, which allows the use of diverse substrates and inocula, adding to its substantial social, economic, and environmental credentials [5]. In dark fermentation, substrates are degraded anaerobically by facultative and obligate anaerobic hydrogen-producing microorganisms. Moreover, this method is an attractive one given that various solid wastes and wastewater can be used as feedstock, thus significantly enhancing process economics while decreasing environmental degradation.

An estimated 200 billion tons of lignocellulosic biomass is produced annually, and it is considered a low-cost and eco-friendly alternative feedstock for high-value products such as biofuels [6]. In addition, lignocellulosic biomass is rich in fermentable carbohydrates, which makes it an attractive feedstock for biofuel production. Sugarcane is one such example, and it is estimated that 1.6 billion tons are produced annually [7]. The leaf component of the sugarcane, comprising up to approximately 40% of the plant, is disposed of prior to harvest by burning. This process releases harmful mutagenic polycyclic aromatic hydrocarbons into the atmosphere, which can have severe effects on human health [8,9]. Due to the complex structure of lignocellulosic materials, a pretreatment is essential, because this allows the breakdown of the cross-linked matrix and promotes the release of fermentable sugars [10]. In a previous study, the modeling and optimization of fermentable sugar release (xylose and glucose) was reported [11]. However, investigations regarding the optimization of key operational parameters for biohydrogen production from sugarcane leaf feedstock are scarce [12].

The physicochemical parameters for biohydrogen production, such as substrate concentration, HRT, pH, temperature, and inoculum concentration impact on the cell metabolism fluxes. The optimization of these parameters is essential to achieve higher hydrogen production [5,13]. The substrate concentration directly affects the formation of volatile fatty acids (VFAs), consequently affecting process pH and ultimately affecting microbial community composition [14]. HRT control in biohydrogen production processes allows the inhibition of hydrogen-consuming microorganisms. In

addition, HRT must be optimal (higher than the growth rate of hydrogen-producing microorganisms) to prevent washout of the biomass [15]. Furthermore, longer HRTs allow the accumulation of VFAs, thus lowering fermentation pH and impeding hydrogen production [13]. The inoculum concentration is another key parameter that has been shown to enhance hydrogen production with increasing concentrations while concomitantly inhibiting methanogens [16]. However, high inoculum concentrations have been found to increase biomass accumulation, which leads to rapid nutrient consumption and waste production [17]. Bioprocess modeling and optimization is required to determine the optimal setpoints of key operational parameters.

Response surface methodology (RSM) is a mathematics- and statistics-based modeling tool. In statistical modeling, deterministic models allow researchers to project and define the dynamics of a system over a measurable period, and this type of model extrapolates the effect of the considered input parameters on the response output [18]. RSM has been employed in the modeling and optimization of various bioprocesses [4,18,19].

This study models and optimizes the physico-chemical input parameters for biohydrogen production from waste sugarcane leaves and assesses hydrogen production on a semi-pilot scale under the optimized operational conditions.

2. Materials and methods

2.1 Inoculum preparation

The anaerobic sludge used in this study was collected from the Darville Wastewater Treatment facility in Pietermartizburg, South Africa. The sludge was immediately transported to the laboratory and stored at 4 °C. Prior to fermentation, it was thermally treated at 121 °C for 10 min to maximize hydrogen production by inhibiting methanogens.

2.2 Feedstock pretreatment

The sugarcane leaves were collected from the South African Sugarcane Research Institute (SASRI), dried at 60 °C for 72 hr and milled using a centrifugal mill (Retsch ZM-1, Durban, South Africa). The sugarcane leaves were optimally pretreated according to the previously established protocol (Chapter 3). This included 20 ml of 4.90% HCl added to 9.45 g of milled sugarcane leaves in a 250 ml Schott bottle. The contents were mixed and heated for 84 min at 99 °C using a PolyScience Analogue water bath. Timing was initiated once the setpoint temperature of the substrate had been reached. The

pretreated solution was adjusted to pH 7 using 10 M NaOH. A process flow diagram illustrating the conversion of sugarcane leaves into biohydrogen is shown in Figure 1.

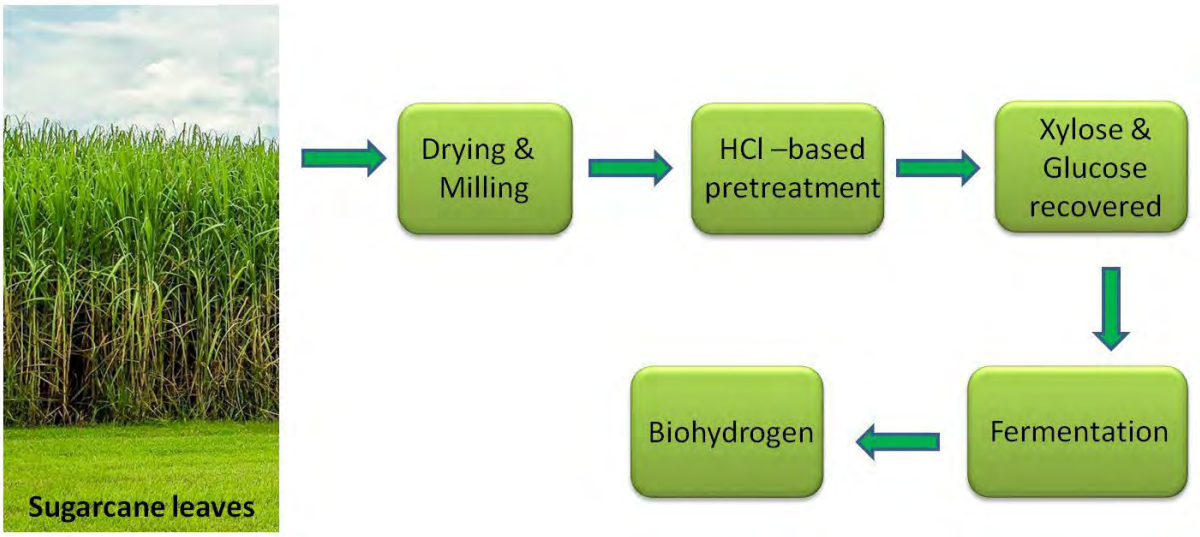

Figure 1. Overview of process flow diagram for the conversion of sugarcane leaves into biohydrogen.

2.3 Experimental design

The RSM Box-Behnken design was used to model and optimize three physico-chemical input parameters for the production of biohydrogen. Three independent variables, namely substrate concentration (A), inoculum concentration (B), and hydraulic retention time (HRT) (C), were considered; their ranges were 8 to 24 (g/l), 10 to 50% (v/v) and 24 to 96 (hr) respectively. Seventeen experimental runs were generated and carried out in duplicate (Table 1).

2.4 Batch fermentation

Fermentation experiments were carried out in modified 250 ml Erlenmeyer flasks with a working volume of 200 ml. All flasks were inoculated with anaerobic sludge and fed with varied volumes of pretreated sugarcane leaves and mineral salts to obtain a final substrate concentration as specified in the design. The supplemented mineral salts comprised (g/l): NH_4Cl 0.5, KH_2PO_4 0.5, K_2HPO_4 0.5, $NaHCO_3$ 4.0, $FeCl_3$ 0.15, $MgCl.6H_20$ 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, and $Na_2MoO_2.2H_2O$ 0.03. Prior to fermentation, the initial pH was adjusted to 6.5 using 1 M NaOH and 1 M HCl, and the flasks were flushed with nitrogen gas for 2 min and then tightly capped with rubber stoppers to promote anaerobiosis, as recommended by Van Ginkel et al [20]. The fermentation processes were run at 37 °C at 180 rpm.

The semi-pilot scale process was carried out in a 13 L bioreactor (Labfors-INFORS HT, Switzerland) with an 8 L working volume. The vessel was fed with 2600 ml pretreated waste sugarcane leaves (WSCL) and 2788 ml of mineral salts, and inoculated with 2612 ml of thermally treated anaerobic sludge. The optimum operational setpoints of 14.23 g/L substrate concentration, 32.68% inoculum concentration, and 62.77 hr HRT obtained in the modeling and optimization phase were applied to the bioreactor. The semi-pilot scale assessment was carried out in duplicate.

2.5. Process monitoring

The semi-pilot scale-up fermentation system was interfaced with the F-Lab monitoring software [21] that allowed online monitoring of the gas evolution. The evolving gas fraction was monitored in real time, with the sampling interval set to 1 min. The cumulative biogas volume was measured using a milligas counter (MGC, Bluesens, Germany). Aliquots from the process was sampled every 6 hr and analyzed for pH change and xylose and glucose consumption.

Table 1 – Box-Behnken design with observed hydrogen yield.

Run	Substrate Concentration (g/l)		Inoculum Concentration (%, v/v)		HRT (hr)		H_2 yield (ml/g sugar)
	A	Code	B	Code	C	Code	
1	24.00	+1	50.00	+1	60.00	0	0.0098
2	16.00	0	10.00	-1	96.00	+1	5.62
3	16.00	0	10.00	-1	24.00	-1	4.01
4	8.00	-1	30.00	0	24.00	-1	0
5	24.00	+1	30.00	0	96.00	+1	0
6	24.00	+1	10.00	-1	60.00	0	0.073
7	24.00	+1	30.00	0	24.00	-1	0
8	16.00	0	50.00	+1	24.00	-1	5.95
9	16.00	0	30.00	0	60.00	0	12.62
10	16.00	0	30.00	0	60.00	0	13.41
11	16.00	0	30.00	0	60.00	0	12.94
12	16.00	0	30.00	0	60.00	0	13.56
13	16.00	0	30.00	0	60.00	0	8.45
14	16.00	0	50.00	+1	96.00	+1	5.35
15	8.00	-1	10.00	-1	60.00	0	7.34
16	8.00	-1	50.00	+1	60.00	0	9.25
17	8.00	-1	30.00	0	96.00	+1	4.04

2.6 Analytical methods

Design Expert (Stat-Ease, USA) was used for model generation and process optimization. The volume of the evolved gas during fermentation was monitored using the water displacement technique. In addition, the fraction of hydrogen was analyzed using a hydrogen sensor (BCP-H_2; Bluesens, Germany) that employs the thermal conductivity measuring principle with measuring ranges of 0 to 100 vol. %. The cumulative volume of hydrogen 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 $V_{H,i-1}$ represent the cumulative hydrogen gas volumes at the current (i) and previous (i-1) time intervals, $V_{G,i}$ and $V_{G,i-1}$ the total biogas volumes in the current and previous time intervals, $C_{H,i}$ and $C_{H,i-1}$ the fraction of hydrogen gas in the headspace of the reactor flask in the current and previous time intervals, and V_H the total volume of headspace in the reactor. Volatile fatty acids (VFAs) were quantified using gas chromatography–FID as described by Faloye et al. [4]. Fermentable sugar was quantified using the YSI Biochemistry Analyzer (Model 2700, select-dual configuration, YSI, USA).

2.7 Bacterial community analysis

DNA was extracted using a modified protocol previously described by Orsini and Romano-Spica [22]. One ml of sample was collected during peak production and suspended in 1 ml of extraction buffer (50 mM Tris-HCl, 25 mM EDTA, 0.1% (w/v) SDS, 0.1% (w/v) PVP, pH 8.0). The samples were centrifuged at 8000 rpm for 1 min, followed by supernatant removal and pellet suspension in 500 µl of lysis buffer (50 mM Tris-HCl, 25 mM EDTA, 3.0% (w/v) SDS, 1.0% (w/v) PVP, pH 8.0). Tubes were then heated at 90 °C for 10 min and rapidly cooled in liquid nitrogen. Pre-warmed (65 °C) extraction solution (500 µl; 10 mM Tris-HCl, 1 mM EDTA, 300 mM sodium acetate, 1.0% (w/v) PVP) was added to the tube. This was followed by the addition of phenol:choloroform:isoamyalcohol (25:24:1) mixed by inversion, and DNA precipitation was achieved using isopropanol. The DNA pellets were washed with 70% ethanol and resuspended in 100 µl TE buffer (pH 8.0). DNA quantification and purity were checked using the Nanodrop 2000 spectrophotometer (Thermo Scientific, USA).

The 16S rRNA gene fragment of extracted DNA was amplified by PCR using the universal bacterial primer 907R (5'-CCGTCAATTCMTTTGAGTTT-3') [23]. The 25µl reactions contained 2 µl $MgCl_2$, 3 µl of 2mM dNTP mix, 3 µl PCR buffer, 0.25 µl of the primer, 0.2 µl Taq polymerase (5u/µl), 1 µl of DNA sample and 15.55 µl nuclease free water. PCR was carried out using the following protocol: initial denaturation at 94 °C for 2 min, 30 cycles of 94 °C for 15 s, annealing at 53 °C for 15 s, and elongation at 68 °C for 25 s, with a final extension step of 68 °C for 5 min. Amplicons were resolved on a 2% agarose gel stained with SYBR Safe, and amplicon sizes were verified using a 1 Kb DNA ladder (Thermo Scientific, USA). The PCR products were subsequently ligated into the pMiniT vector and transformed into competent *E.coli* (NEB 10-beta) cells using the NEB PCR Cloning Kit (New England BioLabs) as per the manufacturer's instructions. Inserts were validated by PCR using the specific forward primer (5′-ACCTGCCAACCAAAGCGAGAAC-3′) and reverse primer (5′-TCAGGGTTATTGTCTCATGAGCG-3′) with PCR conditions described above. Positive clones were

selected for sequence analysis at Inqaba Biotec (Pretoria, South Africa). The sequence results were compared with the available sequences in the NCBI database using the BLAST algorithm.

3 Results and discussion

3.1 Significance of ANOVA

ANOVA was performed to assess the significance of fit for the quadratic model (Table 2). The effect of the input variables on the hydrogen yield is shown in the regression Equation 2:

$$H_2 \text{ yield} = +12.20 -2.57A +0.44B +0.63C -0.49AB -1.01\ AC -0.55BC - 6.13A^2 - 1.90B^2 - 5.06C^2 \quad (2)$$

The p-value is an indicator of the significance of each coefficient. Higher significance is inferred with larger F-values and smaller p-values [18]. The quadratic model fit was significant given an F-value and p-value of 7.46 and 0.0074 respectively. A large F-value is an indication that response variations can be interpreted by the regression equation [18]. The coefficient of determination (R^2) was 0.90, thereby inferring the model's ability to explain 90% of variations in the data. As the R^2 value approaches 1.00, the model can predict the response with a higher degree of accuracy. The p-values also indicate a statistically significant fit for the model, as values <0.01 are generally considered significant [24].

Table 2. ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	Df	Mean square	F-value	P-value
Model	371.09	9	41.23	7.46	0.0074
A	52.7	1	52.77	9.55	0.0176
B	1.55	1	1.55	0.28	0.6132
C	3.19	1	3.19	0.558	0.4723
AB	0.97	1	0.97	0.18	0.6873
AC	4.08	1	4.08	0.74	0.4186
BC	1.22	1	1.22	0.22	0.6526
A^2	157.97	1	157.97	28.59	0.0011
B^2	15.24	1	15.24	2.76	0.1407
C^2	107.84	1	107.84	19.52	0.0031
Lack of fit	20.58	3	6.86	1.52	0.3395

3.2 Interactive effect of input parameters substrate concentration, inoculum concentration, and HRT on H_2 yield

The hydrogen yield obtained for each experimental run is shown in Table 1. From the data, the hydrogen yield is shown to range from 0 to 13.56 ml H_2 g^{-1} fermentable sugar (FS), thus highlighting the sensitivity of hydrogen production to the considered input parameters.

The response surface plots describing the regression model were generated from the deterministic equation (2). These graphs allow an evaluation of the interaction between input parameters. Figures 2A–C indicate that optimum conditions for hydrogen production are located within the design boundary, as shown by the clear peak.

At high substrate concentrations (above 16 g/l; runs 1, 5, 6, and 7), a low yield of hydrogen (< 0.073 ml H_2 g^{-1} FS) was obtained. Figure 2A indicates that a high hydrogen yield was obtained with a substrate concentration in the range of 13 to 20 g/L. Wang and Wan [24] reported an optimum hydrogen yield of 298.8 ml/g with 20 g/L of glucose. Increasing substrate concentration from 8 to 14 g/L while maintaining inoculum concentration at 30%, enhanced hydrogen yield from 8.6 to 12.45 ml H_2 g^{-1} FS. A similar yield pattern can be observed in Figure 2B. An increase in substrate concentration from 8 to 14 g/L while maintaining the HRT at 65 hr enhanced hydrogen yield from 8.8 to 12.48 ml H_2 g^{-1} FS. A simultaneous increase in substrate concentration and inoculum concentration from 8 to 16 g/L and 10 to 30% respectively resulted in an increase in hydrogen yield from 5.80 to 12.19 ml H_2 g^{-1} FS.

High substrate concentration has been reported to cause an overload scenario where minimal or no hydrogen is produced, while low substrate concentration causes decreased microbial metabolic activity resulting in a low hydrogen yield [25]. The highest hydrogen yield was obtained using 16 g/l substrate concentration (run 12). This result is consistent with the studies by Lin and Cheng [26]. These authors reported an optimal substrate (xylose) concentration of 20 g COD/l from a range of 10 to 100 g COD/l.

As shown in Figure 2A, an increase in inoculum concentration from 10 to 33 % while maintaining substrate concentration at 16 g/L resulted in a hydrogen yield increase from 9.85 to 12.21 ml H_2 g^{-1} FS. Similarly, an increase in inoculum concentration from 10 to 32% (Figure 2C) while maintaining HRT at 60 hr increases the hydrogen yield from 9.85 g/L to 12.2 ml H_2 g^{-1} FS. However, increasing inoculum concentration beyond the 33% threshold resulted in a hydrogen yield lower than the maximum yield of 12.5 ml H_2 g^{-1} FS (Figure 2C). A simultaneous increase in inoculum concentration and HRT from 10 to 30% and 24 to 65 hr respectively improved hydrogen yield from 3.5 to 12.20 ml

H_2 g^{-1} FS, a 348% increase. These findings are in agreement with those obtained by Puad et al. [16]. These authors reported a 20% increase in the hydrogen fraction by increasing inoculum concentration from 10 to 30%. Chaganti et al. [18] obtained an optimal hydrogen yield of 2.4 mol/mol xylose with an inoculum concentration of 1800 VSS (mg/l) and a lower hydrogen yield (1.6 mol H_2/mol xylose) with an inoculum concentration of 1000 VSS (mg/l).

Figure 2B illustrates that by increasing the HRT from 24 to 65 hr while maintaining the substrate concentration at 14 g/L, hydrogen yield could be enhanced from 6.6 to 12.51 ml H_2 g^{-1} FS. The hydraulic retention time beyond 65 hr showed reduced hydrogen production. Similarly, it is shown in Figure 2C that an increase in HRT from 24 to 65 hr while maintaining inoculum concentration at 30% could increase hydrogen yield from 6.51 to 12.48 ml H_2 g^{-1} FS. A concomitant increase in HRT and substrate concentration from 24 to 62 hr and 8 to 14 g/L respectively increased hydrogen yield significantly, from 2 to 12.52 ml H_2 g^{-1} FS (Figure 2B). A low HRT (24 hr) has shown reduced hydrogen yields, whereas maximum hydrogen is produced at 65 hr. Low hydrogen production rates have been linked to low HRT, as previously reported by Lay [19]. Complex substrates such as food waste and sewage biosolids have shown to require longer optimum HRTs (36 and 24 hr respectively) compared to glucose and sucrose, which require 2 and 6 hr respectively [13].

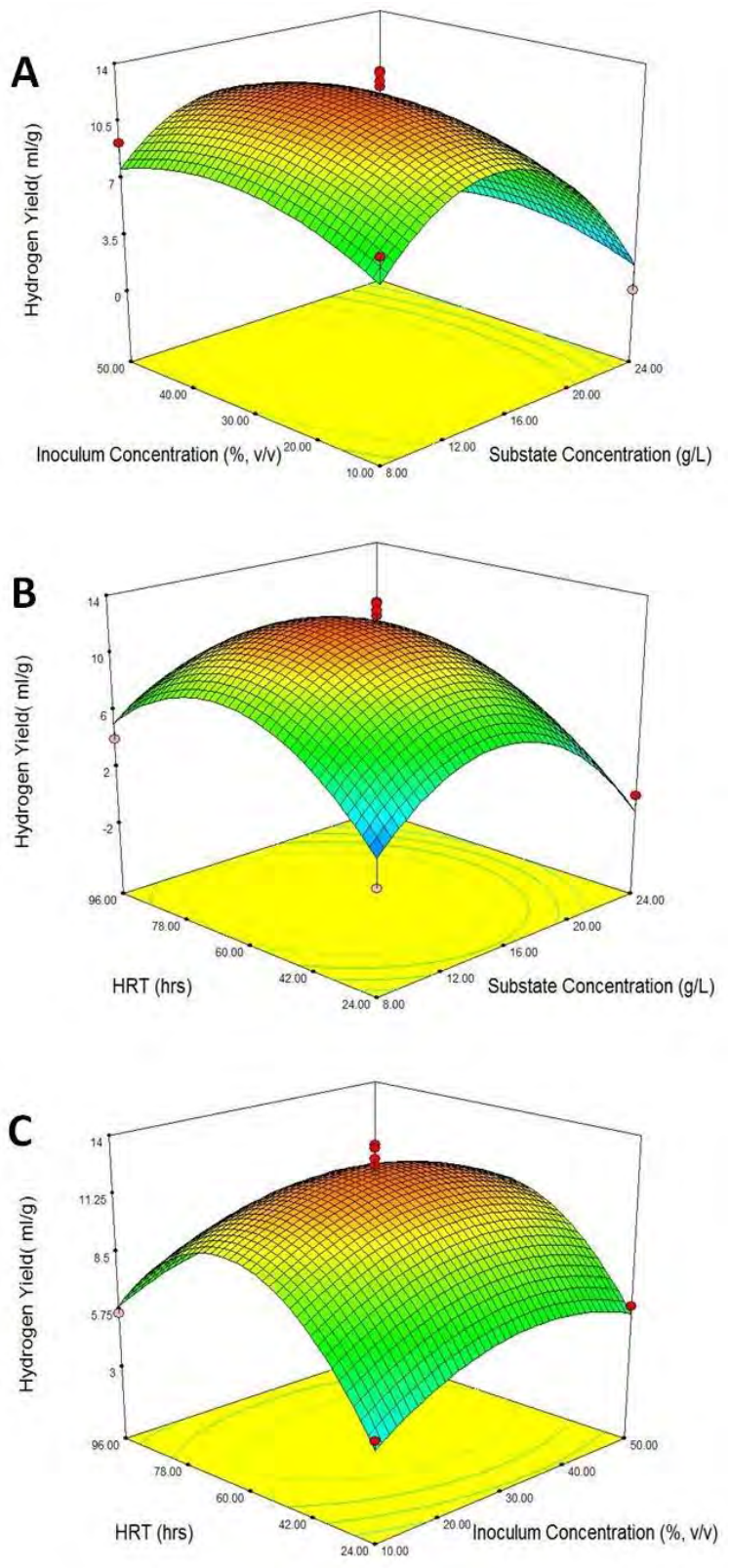

Figure 2. Response surface plots showing interactive effect of (A) inoculum and substrate concentration, (B) HRT and substrate concentration, and (C) HRT and inoculum concentration on hydrogen yield.

3.3 Validation of optimized process condition

The model predicted a maximum yield of 12.5 ml H_2 g^{-1} FS under optimized operational conditions of 14.23 g/L substrate concentration, 32.68% inoculum concentration, and 62.77 hr HRT. The experimental validation of these setpoints carried out in triplicate gave 12.76 ± 0.65 ml H_2 g^{-1} FS, thus a negligible prediction error of 5% was observed. The optimized hydrogen yield showed a 45% improvement from the non-optimized hydrogen run, which produced 7.05 ml H_2 g^{-1} FS. These optimized operational conditions were subsequently used for the semi- pilot scale assessment.

3.4 Semi-pilot scale-up of H_2 production

The semi-pilot process showed a lag phase lasting 23 hr, which is similar to the 20 h lag phase observed in the bioconversion of wheat straw to biohydrogen by Fan et al. [27]. A lengthy lag phase can be attributed to the reactor size and the nature of the substrate. For instance, Lin et al. [28] observed a lag phase of 9 days using a 400 L reactor with sucrose medium, whereas Sekoai and Kana [29] reported a lag phase of 5 hr in a 10 L reactor.

Figure 3 shows that glucose and xylose metabolism began simultaneously, with glucose being rapidly depleted within 7 hr of fermentation from 1.7 to 0 g/L. Microbial cells have a preferential affinity for glucose consumption over xylose [11]. Xylose degradation occurs either via the acetate or butyrate pathway; however, mixed cultures have been shown to produce hydrogen using both metabolic pathways [30]. Hydrogen production began at 24 hr and peaked at 37% at approximately 66 hr, thus an exponential phase of 42 hr exists. A total cumulative volume of 3739.95 ml H_2 was obtained and a yield of 321 ml H_2 g^{-1} FS, corresponding to 49.87 ml H_2 g^{-1} feedstock.

Cui et al. [31] reported a cumulative hydrogen yield of 34 ml with an exponential phase lasting approximately 20 hr with acid-pretreated poplar leaves. In another study, hydrogen production from sugarcane bagasse hydrolysate showed an exponential phase lasting 130 hr and a cumulative hydrogen volume of 1350 ml/L [32]. Compared to our previous investigation, which produced a hydrogen yield of 248 ml H_2 g^{-1} FS [11], the modeling and optimization of operational parameters of substrate concentration, inoculum concentration, and HRT in this study enhanced the hydrogen yield by 23%.

The VFA profile indicated that both degradation pathways occurred, with sugar conversion to acetate being the favored pathway; thus, acetic acid was the major VFA detected, followed by butyric acid, at 306 and 78.74 mg/100 ml respectively (Table 3). *Clostridium beijerinckii* and *Clostridium bifermentans* have shown to produce biohydrogen accompanied with acetate and butyrate as the major

by-products [33,34]. Moreover, *Enterobacter cloacae* have also been shown to produce both acetic and butyric acid as by-products in hydrogen fermentation, with acetic acid being the major byproduct in lowering the culture's pH [35]. During hydrogen production, *Klebsiella* sp. produces succinic and acetic acid with ethanol [36].

Hydrogen production declined when the pH decreased to 5.0 at the 65th hr of fermentation. This pH drift was attributed to the accumulation of VFA products, which lowered the pH and ultimately reduced the buffering capacity of the medium, thus promoting unfavorable conditions for biohydrogen production, as suggested in the study conducted by Guo et al. [37]. Similar trends were described by Chaganti et al. [18], who reported a decrease in hydrogen yield from 1.6 to 0.3 $mol.mol^{-1}$ xylose when the pH dropped from 7.6 to 5.0. A pH decrease causes acidogenic processes to shift to solventogenic processes, thus producing mostly acetone, butanol, and ethanol, which inhibit hydrogen production [38].

Table 3. Concentration of measured VFAs

Substrate	Volatile fatty acid (mg/100 ml)			
	Acetic	Butyric	Iso-butyric	Propionic
WSCL*	306	7.74	71.0	0.00

*WSCL – Waste sugarcane leaves

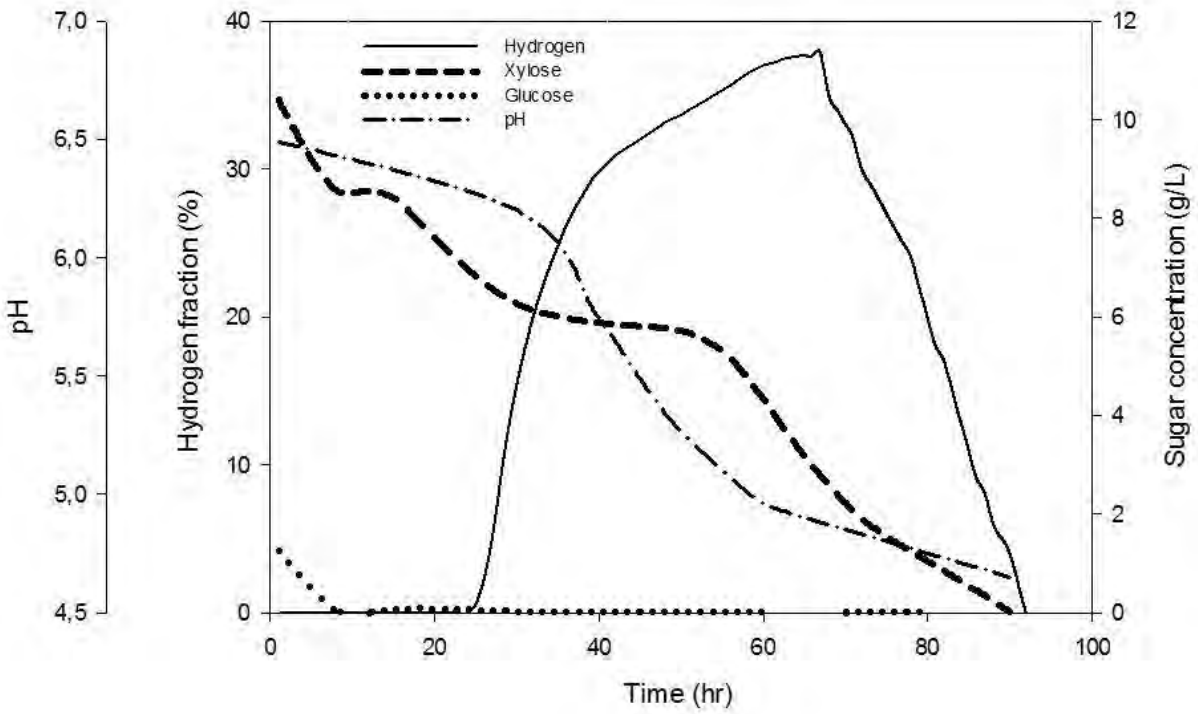

Figure 3. Time course of hydrogen production, sugar consumption, and pH change for the semi-pilot scale-up process.

3.5 Microbial analysis

Analysis of the microbial diversity revealed the presence of *Clostridium* sp., *Klebsiella* sp., and *Enterobacter* sp. (Table 4). These microorganisms have been previously reported as the major hydrogen producers in a sludge inoculum system [39], and the results are similar to those reported by Song et al. [40]. These authors employed thermal treatment on a mixed consortium and reported a microbial community dominated by *Clostridium* sp. and *Enterobacter* sp. *Clostridium beijerinckii* and *Clostridium bifermentans* are endospore forming, gram-positive bacteria and likely to survive the heat pretreatment during the inoculum preparation [41]. Moreover, *Clostridium beijerinckii* and *Clostridium bifermentans* have been reported to produce large amounts of hydrogen (311.3 ml H_2 Lh^{-1} and 2.1 mmol-H_2/g COD respectively) using xylose and wastewater sludge substrates [33,42].

Kraemer and Bagley [39] observed that not all non-spore forming cells were killed by heat treatment, which explains the detection of non-spore formers such as *Klebsiella* sp. and *Enterobacter* sp. This rationale was confirmed by Iyer et al. [43], where after heat treatment at 105 °C for 2 hr species belonging to the *Enterobacter* and *Klebsiella* genera were detected. Owing to their rapid growth rate and efficient substrate consumption, facultative anaerobes often dominate microbial communities during fermentation, thus outcompeting other microbial species [44]. However, their presence may be

beneficial for consuming residual oxygen in the reactor [39]. It is proposed that these groups of microorganisms are responsible for hydrogen production via the formate cleavage pathway [44]. The hydrogen producing efficiency of *Enterobacter cloacae* was demonstrated by Sun et al. [45], who reported a yield of 707 ml H_2/L.

Table 4. Phylogenetic affiliation of 16S rDNA gene sequencing from cloning analysis

Microorganism	Access number (NCBI)	Similarity %
Clostridium beijerinckii	NR_029230.1	99
Clostridium bifermentans	NR_119066.1	98
Enterobacter cloacae	NR_102794.1	99
Klebsiella oxytoca	NR_102982.1	99
Klebsiella pneumoniae	NR_074913.1	98

3.6 Yield comparison

A comparative assessment of the yield obtained using other lignocellulosic feedstocks is presented in Table 5. Zheng et al. [46] reported on the enzymatic pretreatment of poplar leaves and obtained 44.92 ml H_2/g feedstock; however, enzymes incur additional costs, thus reducing commercial feasibility. Significantly lower yields were observed for rice straw and barley hulls (24.8 and 29.2 respectively). The observed low yield could be attributed to the low release of fermentable sugar due to a lack of substrate pretreatment. These data show that more hydrogen can be produced from low-cost sugarcane leaves than other agricultural residues. The order of hydrogen yield from various lignocellulosic feedstocks, as shown in Table 5, increases from rice straw to soybean straw.

Table 5. Hydrogen yield from different lignocellulosic feedstocks

Feedstock	Hydrogen Yield (ml H_2/g feedstock)	Reference
Rice Straw	24.8	Chen et al. [47]
Barley hulls	29.2	Magnusson et al. [48]
Soybean straw	60.2	Han et al. [49]
Grass	39.5	Cui and Shen [50]
Poplar leaves	44.92	Cui et al. [31]
Sugarcane leaves	49.87	This study

4. Conclusion

This study modeled and optimized the key operational parameters of substrate concentration, inoculum concentration, and HRT for biohydrogen production using pretreated waste sugarcane leaves. The developed model produced an R^2 value of 0.90, with a hydrogen yield of 12.5 ml H_2/g FS under optimized conditions (14.23 g/L substrate concentration, 32.68% inoculum concentration, and 62.77 hr HRT). A semi-pilot scale assessment under optimized operational conditions produced a biohydrogen yield of 321 ml H_2 g^{-1} FS, which was a 23 % improvement compared to the unoptimized process. Sugarcane leaves were shown to be a feasible substrate for biohydrogen production compared to other agricultural residues, given the significantly higher hydrogen yield. Furthermore, the scalability of this process was demonstrated on a semi-pilot scale. These findings highlight an alternative route for managing waste sugarcane leaves by using them in the production of biohydrogen.

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Chapter 5

Techno-economic analysis of a large scale production of biohydrogen from waste sugarcane leaves using dark fermentation

Abstract

A techno-economic analysis of commercial production of biohydrogen using dark fermentation from waste sugarcane leaves is reported. The baseline plant capacity was 1.4 $\times 10^6$ L hydrogen/year. The simulation was based on data generated from optimization studies. Waste sugarcane leaves were subjected to heat and acidic treatment. Fermentation was carried out by a mixed microbial consortium via the acetate pathway, and biohydrogen was recovered. The initial capital investment was \$4.9 x 10^6. Facility-dependent costs comprised 64% of the annual operating costs and the raw materials 17%. The unit production cost was \$0.96/L hydrogen. A gross margin of 20% with an annual net profit of \$6.47 $\times$ 10^5 was obtained with a selling price of \$1.2/L. Uncertainty analysis showed that the unit production cost decreased as the plant capacity increased. These findings provide data for strategic R&D investment, and early stage knowledge on economic viability of biohydrogen production from waste sugarcane leaves.

Keywords: Sugarcane leaves, biohydrogen, economic analysis, cost estimate, sensitivity analysis

1. Introduction

Fossil fuels such as crude oil and natural gas dominate the world energy market, which is worth upwards of 1.5 trillion dollars (Goldemberg 2006). There is major uncertainty surrounding the supply of fossil fuels in the near future thus the cost of crude oil is projected to increase from $56/barrel in 2015 to $141/barrel in 2040 and natural gas from $3.73/British thermal units (Btu) in 2013 to $7.85/Btu in 2040 (EIA 2015). The significant increase in fuel prices is associated with the projected exhaustion of oil and gas by 2042 and coal by 2112 (Shahriar and Topal 2009). In addition, a consequence of fossil fuel combustion is the increased emission of greenhouse gases that has resulted in a global mean temperature increase of 0.8 ^{o}C in the last century. This has negatively affected weather and climate patterns as well as various ecosystems, thus, also majorly contributing to the exploration of renewable energy sources (Kothari et al. 2012).

The above scenario has prompted research towards renewable energy technologies. From the array of renewable energy options, hydrogen is one such alternative that is garnering significant interest since its high energy content (122 kJ/g) is 2.75 times greater than conventional hydrocarbon fuels (Faloye et al. 2014). In the near future, hydrogen will be a major factor in the global energy market. The combustion of hydrogen gas yields only water as a by-product and as a result mitigates the production of pollutants (Faloye et al. 2014).

The production of hydrogen via biological pathways appears to be more profitable compared to other methods, thus attracting major research (Ghimire et al. 2015). Among the various biological methods employed to produce hydrogen, dark fermentation has the added benefits of being able to utilize various wastes such as agricultural or municipal wastes and does not require light, thereby continuously producing hydrogen. Agricultural waste such as rapeseed straw, wheat straw and sorghum bagasse has been considered for fermentable sugar production at laboratory scale (Castro et al. 2011, Qureshi et al. 2013, Kamireddy et al. 2013). These lignocellulosic-based materials often require pretreatment prior to fermentation. Depending on the pretreatment regime, the process can often appear economically unfavorable with the employment of enzymatic treatment and other costly, energy intensive techniques (Qureshi et al. 2013). Furthermore, there is a dearth of studies on techno-economic analysis of biohydrogen production at large scale from wastes sugarcane leaves in public repositories.

Sugarcane leaves as agricultural residues are considered as waste and are often disposed of by burning. This practice releases carcinogenic particulate matter into the surrounding environment, which has detrimental health effects (Silva et al. 2010). In recent studies, we described the utilization of xylose and glucose released from these leaves via dilute acid pretreatment for biohydrogen production. The pretreatment released significant fermentable sugar that can be converted into high

value products such as biohydrogen (Moodley and Kana 2015). This highlights the feasibility of using a waste feedstock, which will significantly improve the material costs for the production of biohydrogen. South Africa alone produces more than 20 million tons of sugarcane and approximately 90% is harvested by hand. Since the leaves constitute roughly 40% of the sugarcane plant, this equates to 8 million tons of biomass with the potential to drive South Africa's energy sector towards renewable energy (Smithers 2014). In order to make a commercially viable biohydrogen production process from sugarcane leaves, it is imperative to evaluate the impact of the various input factors involved. These factors include material, utility and equipment costs.

This study aimed at performing a simulation and techno-economic analysis of biohydrogen production from waste sugarcane leaves in a large capacity plant. It further identifies and assesses the sensitivity of unit production cost on key factors affecting the biohydrogen production plant.

2. Materials and methods

2.1. Process description using the base case

Biohydrogen production from waste sugarcane leaves (SCL) was simulated using Superpro Designer, Intelligen, USA. The flow sheet for the simulation is shown in Figure 1. It comprises of the upstream, fermentation and downstream sections. The feedstock was recovered from sugarcane plantations prior to the harvesting season and transported to the plant using a truck (P-5), where it was stored in silos (V-101) and heated for 72 h at 60 ^{o}C to reduce the water content, followed by grinding to a particle size of approximately 1 mm using an industrial grinder (GR-101). The ground leaves were pretreated with 4.98% (v/v) HCl at a solid to liquid ratio of 47.26% (w/v) and heated at 99 ^{o}C for 84 min in a pretreatment reactor (R-101). The pretreated slurry was then cooled to 45 ^{o}C followed by pH adjustment to 6.5 with 1M NaOH. This step ensured the release of fermentable sugar (xylose and glucose). Parallel to this process, sewage sludge containing a mixed microbial population often dominated by spore-formers such as *Clostridium* spp. (Faloye et al. 2014), was heat treated at 121 ^{o}C for 10 min in a storage unit (V-103). This pretreatment ensured the deactivation of hydrogen consuming organisms such as methanogens and acetogens.

The neutralized waste sugarcane leaves slurry was subsequently fed into a batch fermentation reactor (FR-101) and inoculated with heat treated sewage sludge and supplementary mineral salts. The choice of a mixed culture for fermentation from sewage sludge allows an ease of operation since there is a minimal need for sterile conditions allowing a more flexible work flow (Faloye et al. 2014). The

fermentation process within the bioreactor unit FR-101 ran for 65 hours at 37.5 ºC, pH of 6.5 and an agitation rate of 180 rpm. Our previous study has shown that a hydrogen yield of 248 ml/g of fermentable sugar (xylose and glucose) can be obtained from pretreated sugarcane leaves using a mixed microbial culture from sewage sludge (Moodley and Kana 2015).

The evolving hydrogen gas was separated with a Baghouse filter (BHF-101) while the process effluent was treated in a bio-oxidation tank (AB-101) that functioned as an oxidative pond. The Baghouse filter removes any particulate matter from the emitted gas and can allow a selected gas to pass through. The capacity of the simulated base case plant was 1.4×10^6 L of hydrogen/year.

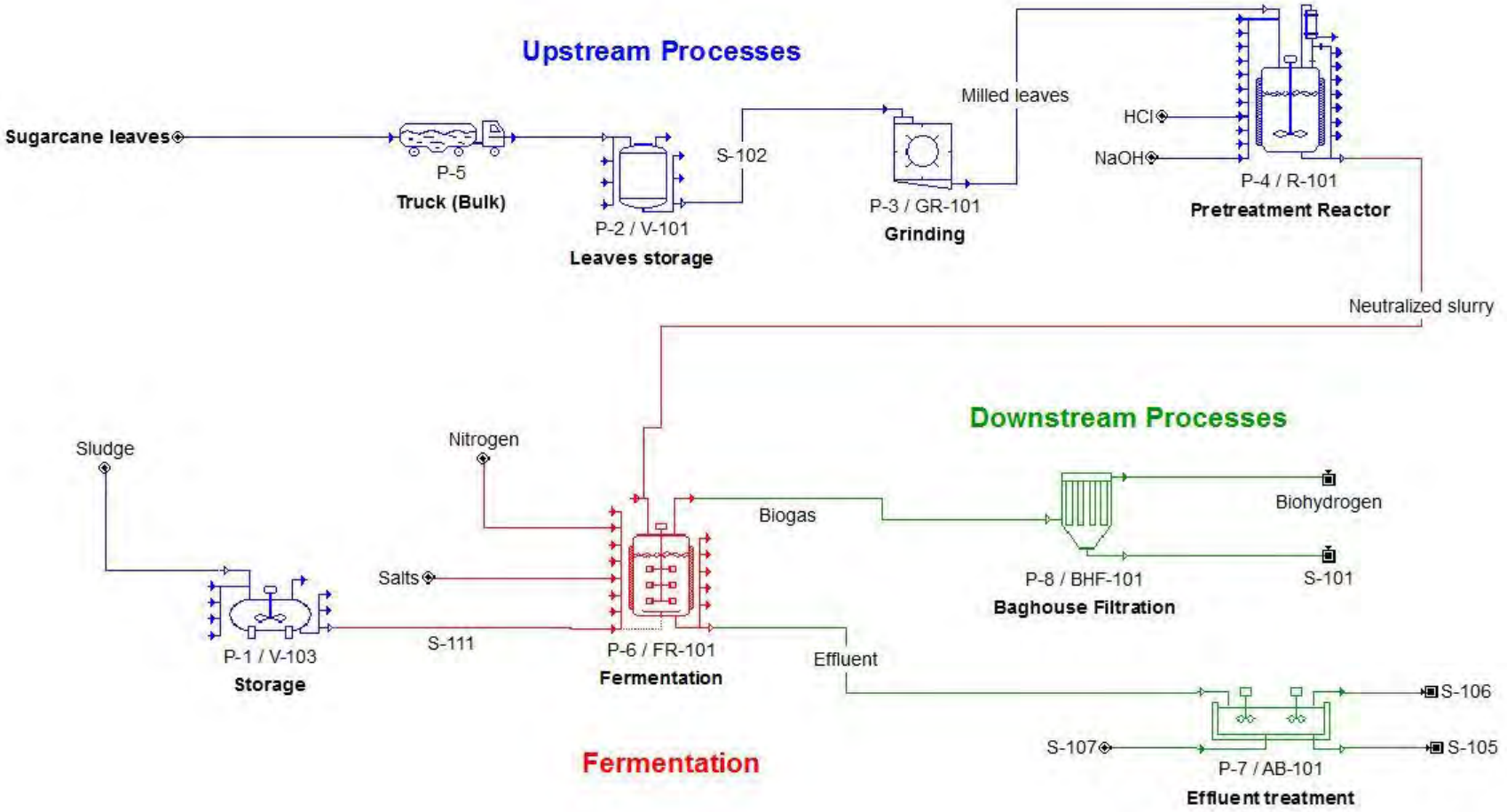

Figure 1. Simulated flow sheet for hydrogen production using waste sugarcane leaves showing the upstream, fermentation and downstream procedures.

2.2 Economic evaluation estimates

The economic evaluation was performed using SuperPro Designer. It is equipped with an updated database of equipment and processes. Equipment prices were based on 2015 pricing, and adjusted according to information provided by local vendors on specific equipment. The simulated base case plant was to be operated for 327 days/year and to have a lifetime of 60 years with straight line depreciation. All costs are expressed as US dollars. The economic estimates for the total capital investment, total product cost and unit production cost were computed in Superpro designer according to equations 1, 2 and 3, respectively (Zhang et al. 2015).

$$TCI = I_{IE} \times (1 + \sum_{i-1}^{n} RF_i) \quad (1)$$

where TCI is total capital investment, I_{IE} is the main equipment costs, RF_i is the ratio factor for direct, indirect and working capital investment, *i* is the items listed in Table 1.

$$TPC = C_{RM} + C_U + C_{L\&F} + C_T \quad (2)$$

where TPC is total product cost, C_{RM} is the raw material cost, C_U is utility cost, $C_{L\&F}$ is labor and facility dependent costs, C_T is transport costs.

$$UPC = \frac{C_{AO}}{Y} \quad (3)$$

where UPC is the unit production cost, C_{AO} is the annual operating costs and Y is the annual product yield.

2.3 Sensitivity analysis

Sensitivity analysis is a technique used to examine how the projected performance varies with changes in key factors on which the projections are based. Thus, it can illustrate the uncertainty associated with fluctuating prices and how this will alter the cost of production (Ong et al. 2012). For this study, sensitivity was carried out to assess the impact of the cost of HCl, and changes in the plant capacity on the unit production cost of biohydrogen.

3. Results and Discussion

3.1 Fixed capital estimate

The fixed capital estimates are shown in Table 1. It comprises the total plant direct cost (TPDC) and the total plant indirect cost (TPIC). For this base case, the equipment costs were estimated at $\$7.63 \times 10^5$ while installation, process piping and instrumentation cost were estimated at $\$3.56 \times 10^5$, $\$2.67 \times 10^5$, and $\$3.05 \times 10^5$, respectively (Table 1). Other factors contributing to the total plant direct cost (TPDC) were buildings, yard improvements and auxiliary facilities which were estimated at $\$3.43 \times 10^5$, $\$1.14 \times 10^5$ and $\$3.05 \times 10^5$, respectively. The total plant indirect cost (TPIC) was projected to be $\$1.53 \times 10^6$ and comprised of engineering and construction. The direct fixed capital cost to start this plant was estimated at $\$4.70 \times 10^6$. Fixed capital for small to medium biofuel plants using lignocellulosic material have been reported in the range of $\$5.4 \times 10^6$ to $\$193 \times 10^6$ (Karmee et al. 2015, Qureshi et al. 2013).

Consideration was taken to select equipment units that could perform various operations, thus reducing the price of multiple equipment units. In this regard, the simulated plant comprised seven major units as outlined in Table 2. The fermentation reactor (FR-101) had the highest cost attached to it ($\$3 \times 10^5$) due to its high volume capacity and instrumentation required. The Baghouse filter was the second costliest unit ($\$9 \times 10^4$) and was justified by its instrumentation and nature of operation. It extracts particulate matter from the evolving gas while selectively allowing hydrogen to pass through, purifying it to some degree. The total equipment costs were projected to be $\$7.6 \times 10^5$.

Table 1. Fixed capital estimate for biohydrogen production from sugarcane leaves (plant capacity 55 × 10^4 kg SCL/year).

A. Total plant direct cost (TPDC)	($)
1. Equipment purchase	763,000
2. Installation	356,000
3. Process piping	267,000
4. Instrumentation	305,000
5. Insulation	23,000
6. Electrical	76,000
7. Buildings	343,000
8. Yard improvements	114,000
9. Auxiliary facilities	305,000
Total plant direct cost (TPDC)	2,552,000
B. Total plant indirect cost (TPIC)	
1. Engineering	638,000
2. Construction	893,000
Total plant indirect cost (TPIC)	1,531,000
C. Total plant cost (TPC = TPDC+TPIC)	4,084,000
D. Contractor's fee and contingency (CFC)	
1. Contractor's fee	204,000
2. Contingency	408,000
Total contractor's fee and contingency (CFC)	613,000
E. Direct fixed capital cost (DFC= TPC+CFC)	4,696,000

Table 2. Equipment costs for biohydrogen production from sugarcane leaves (plant capacity 55×10^4 kg SCL/year).

Item code	Equipment [a]	Description	Quantity	Cost ($)
V-101	Receiver tank	Vessel volume = 45000.00 L	1	50,000
GR-101	Grinder	Capacity = 2646.60 kg/h	1	20,000
R-101	Stirred reactor	Vessel volume = 13000.00 L	1	50,000
FR-101	Fermenter	Vessel volume = 40883.67 L	1	300,000
V-103	Horizontal with mixer tank	Vessel volume = 7255.56 L	2	50,000
BHF-101	Baghouse filter	Total bag area = 714.14m^2	1	90,000
AB-101	Aeration basin	Vessel volume = 8308.40 L	1	50,000
		Unlisted equipment		153,000
			Total	763,000

[a] Equipment is manufactured with stainless steel SS316

3.2 Material, utility and annual operating cost estimates

These are the costs of producing the biohydrogen product, and include the materials, utilities, labor cost, consumables, facility-dependent cost, laboratory cost, transportation and waste-treatment. Table 3 shows a breakdown of the material costs. Annually, 5.5×10^5 kg of sugarcane leaves will be processed. These leaves will be collected from sugarcane fields pre-harvest, thus eliciting negligible cost. Items that greatly impact the annual material budget of the plant are HCl, $FeCl_3$, K_2HPO_4, KH_2PO_4, $Ca(OH)_2$ and NH_4Cl, which have costs of $\$6.6 \times 10^4$, $\$ 12 \times 10^3$, $\$4.2 \times 10^4$, $\$3.9 \times 10^4$, $\$2 \times 10^3$ and $\$3.9 \times 10^4$, respectively. A high quantity of HCl is required for the pretreatment phase of the production process, and the NaOH is used as a neutralizing agent for the pretreated slurry. A successive enzymatic saccharification step was not considered for this process as it has been demonstrated in our previous study that fermentable sugar at a concentration of 89.48 g/L can be recovered from sugarcane leaves with sole acid pretreatment. Some reported studies on lignocellulosic pretreatment with acid have considered a second step of enzymatic hydrolysis using 10FPU/g of Celluclast and 200 nkat/g of Novozyme 188 (Yang et al. 2013). These enzymes cost between $100 and $200 (Sigma-Aldrich), thus impacting significantly on material cost at pilot scale. Since water is an integral component to many operations in this process, high quantities (1.5×10^6 kg/year) will be required annually. Therefore, the total annual cost for materials is $\$2.37 \times 10^5$.

Standard power, steam and chilled water are three main utilities for this plant (Table 4). Several unit procedures require significant heating for prolonged periods. These include Storage tank V-101 to dry the leaves at 60 ^{o}C, pretreatment reactor R-101 pretreating the leaves at 99 ^{o}C, fermentation reactor FR-101 maintaining the temperature at 37.5 ^{o}C and storage tank V-103 treating the sludge at 121 ^{o}C. Annually, these utilities amount to 8.69×10^5 kW-h, 529 MT and 1.48×10^5 MT, respectively, with an annual total running cost of $\$1.09 \times 10^5$. By comparison, Qureshi et al. (2013) reported utility costs of $\$93 \times 10^6$, comprising 49% of the annual operating costs. This high utility cost is linked with the requirement for several distillation columns, which were used in the downstream processing of butanol. In addition, chilled water comprised 71% of the utilities at a projected cost of $\$67.79 \times 10^6$/year.

Annual operating costs of the plant are presented in Table 5, which include raw materials, labor-dependent, facility-dependent and utilities with an annual running cost of $\$1.32 \times 10^6$. Facility dependent costs make up 64.57% of the total annual operating cost followed by 17.90% for raw materials. Mel et al. (2015) reported an annual operating cost of $\$11 \times 10^6$ for a biogas plant with facility-dependent and raw materials making up 31% and 49% of this cost, respectively. As confirmed in this study, the facility-dependent cost made up a significant portion of the annual operating costs. Agricultural wastes often have minimal costs, thus making their conversion to biofuel more profitable (Sekoai and Gueguim Kana 2013).

Table 3. Material costs for biohydrogen production from sugarcane leaves (plant capacity 55×10^4 kg SCL/year).

Bulk material	Unit cost ($)	Annual amount		Annual cost ($)	%
Sugarcane leaves	0.000	550,995	kg	0	0.00
HCl (4.98%)	2.000	33,023	L	66,046	27.81
NaOH (10M)	7.000	54	L	379	0.16
Nitrogen	1.500	101	L	152	0.06
$Ca(OH)_2$	0.129	17,188	kg	2,217	0.93
$CaCl_2.2H_2O$	20.00	43	kg	859	0.36
H_3BO_3	52.54	129	kg	6,773	2.85
$FeCl_3$	20.00	645	kg	12,891	5.43
K_2HPO_4	20.00	2,149	kg	42,971	18.09
KH_2PO_4	18.00	2,149	kg	38,674	16.28
$MgCl_2.2H_2O$	47.490	365	kg	17,346	7.30
$MnCl_2.2H_2O$	25.00	129	kg	3,223	1.36
$NaMoO_4.2H_2O$	25.00	129	kg	3,223	1.36
NH_4Cl	18.00	2,149	kg	38,674	16.28
Water	0.0003	1,497,550	kg	449	0.19
$ZnSO_4.7H_2O$	72.780	43	kg	3,127	1.32
Biomass	0.000	1,494,717	kg	0	0.00
Total				237,006	100.00

Table 4. Utility costs for biohydrogen production from sugarcane leaves (plant capacity 55×10^4 kg SCL/year).

Utility and unit	Annual amount	Annual cost ($)	%
Std power (kW-h)	869,347	43,467	39.82
Steam (MT)	529	6,350	5.82
Chilled water (MT)	148,373	59,349	54.37
Total		109,167	100

Table 5. Annual operating cost for biohydrogen production from sugarcane leaves (plant capacity 55×10^4 kg SCL/year).

Cost item	Amount ($)	%
Raw materials	238,000	17.90
Labor-dependent	101,000	7.60
Facility-dependent	857,000	64.57
Utilities	109,000	8.23
Transport	23,000	1.70
Total	1,327,000	100.00

3.3 Profitability analysis

Table 6 presents the profitability analysis for the plant. The initial capital investment required is $\$4.97 \times 10^6$ and encompasses direct fixed capital, working capital and startup cost at $\$4.70 \times 10^6$, $\$41 \times 10^3$ and $\$2.35 \times 10^5$, respectively. The revenue price for biohydrogen production from sugarcane leaves was estimated to be \$1.2/L based on previous and current gasoline and natural gas price trends (EIA 2015), taking into account an inflation rate of 4%. A gross margin of 20.12% and annual net profit of $\$6.47 \times 10^5$ was achieved and thus, a payback time of 7.69 years. Mel et al. (2015) reported a gross

margin of 11.91% with a capital investment and annual revenues of \$6.1 × 10^6 and \$3 × 10^6 respectively for a biogas production plant.

Some considerations are required to interpret these profitability data. It has been shown that, with the development of novel bioprocesses, significant variations may exist with the plant performance (Merrow et al. 1981). These authors demonstrated that technologies that are not yet commercially proven have potentials for unseen problems on design, construction, startup and operations, thus resulting in underestimation of capital cost and overestimation of profit margin. Cognizant of such limitations, it is important to assess the impact of other key model parameters on the sensitivity of the production cost.

Table 6. Profitability analysis for biohydrogen production from sugarcane leaves (plant capacity 55 × 10^4 kg SCL/year).

A. Direct fixed capital (\$)	4,696,000
B. Working capital (\$)	41,000
C. Startup cost (\$)	235,000
D. Total investment (A + B + C) (\$)	4,972,000
E. Investment charged to this project (\$)	4,972,000
F. Biohydrogen production (L/year)	1,384,251
G. Annual operating cost (\$/year)	1,327,000
H. Selling price for biohydrogen (\$/L)	1.20
I. Revenue from biohydrogen (\$/year)	1,661,101
J. Gross profit (I – G) (\$/year)	334,000
K. Taxes (40%) (\$/year)	134,000
L. Net profit (J – K + Depreciation) (\$/year)	647,000

3.4 Sensitivity analysis

Sensitivity analysis was carried out to assess the impact of fractional changes in HCl price and plant capacity on the unit production cost. HCl cost made up 27.81% of the material costs. The impact of changing the HCl price from its base value of $2/L on unit production cost is shown in Figure 2. A linear effect is observed in which a 15 or 20% change from the base value has only $0.01/L impact on unit production cost of biohydrogen. A 10% fractional increase or reduction in HCl price has no effect on the biohydrogen unit production cost using waste sugarcane leaves. These sensitivity data show that an unforeseen price fluctuation of HCl within a 20% range has a negligible effect on the unit production cost of biohydrogen from this feedstock.

Fermentation costs affect the profitability of the fermentation plant. Additionally, the plant capacity is a critical decision variable for investment and management. Thus, it is useful to evaluate the economies of scales at various scales of production. The economies of scale refer to the potential decrease in unit production cost as the scale of production increases. The scaling process of the plant used a scaling exponent function according to Equation 4, whereby a nonlinear cost relationship is used to estimate the cost of new equipment for a different plant size based on a known equipment cost of a different size.

$$\text{Cost 2} = \text{Cost 1} \left(\text{Size 2}/\text{Size 1}\right)^{0.6} \quad (4)$$

In this base case design, the plant's production capacity significantly affected the unit production cost of biohydrogen. Figure 3 illustrates the effect of fractional change in the plant production capacity on the unit production cost. An asymptotic trend was observed where fractional increases in the plant's capacity from -75% to 200% of its base value of 55×10^4 kg sugarcane leaves/year results in a decrease in unit production cost from $4/L to $0.3/L. A 100% expansion of the plant's capacity implies that 11×10^5 kg of leaves will be processed, which will increase the yield to 2,847,266 L/hydrogen annually at a unit production cost of $0.48/L (Table 7). This translates to a 105% increase in biohydrogen yield. A similar trend is observed with a 150 and 200% fractional increase in the plant. Therefore, expanding the plant's capacity resulted in a larger profitable margin since the unit production cost is significantly reduced.

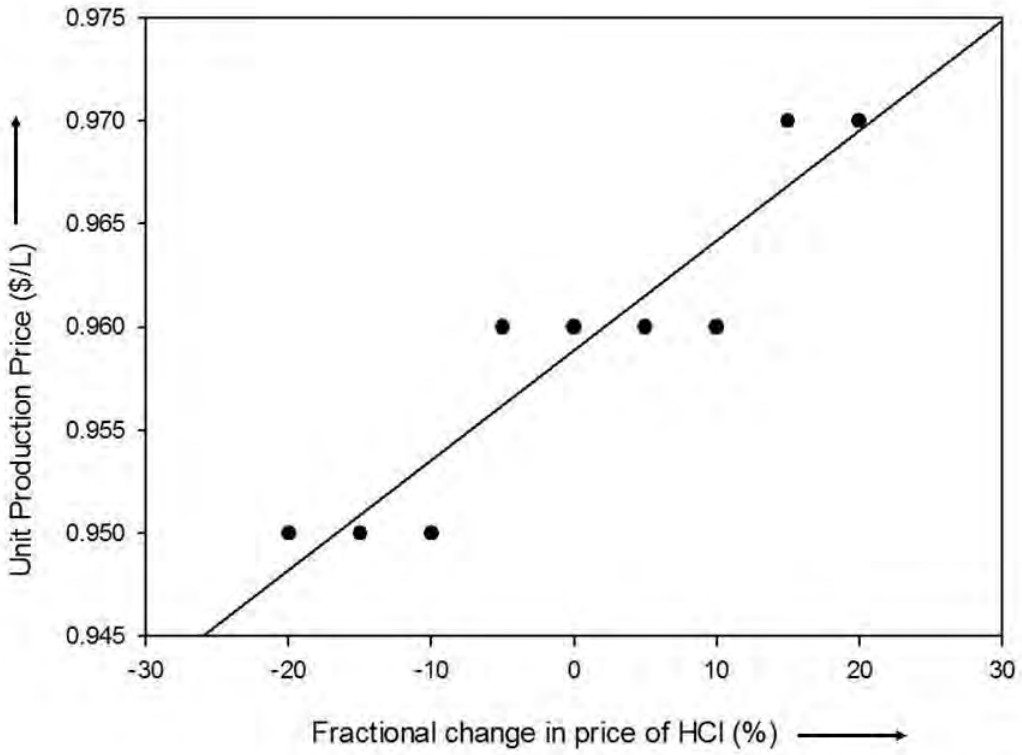

Figure 2. Effect of fractional changes in the price of HCl on the unit production cost of biohydrogen.

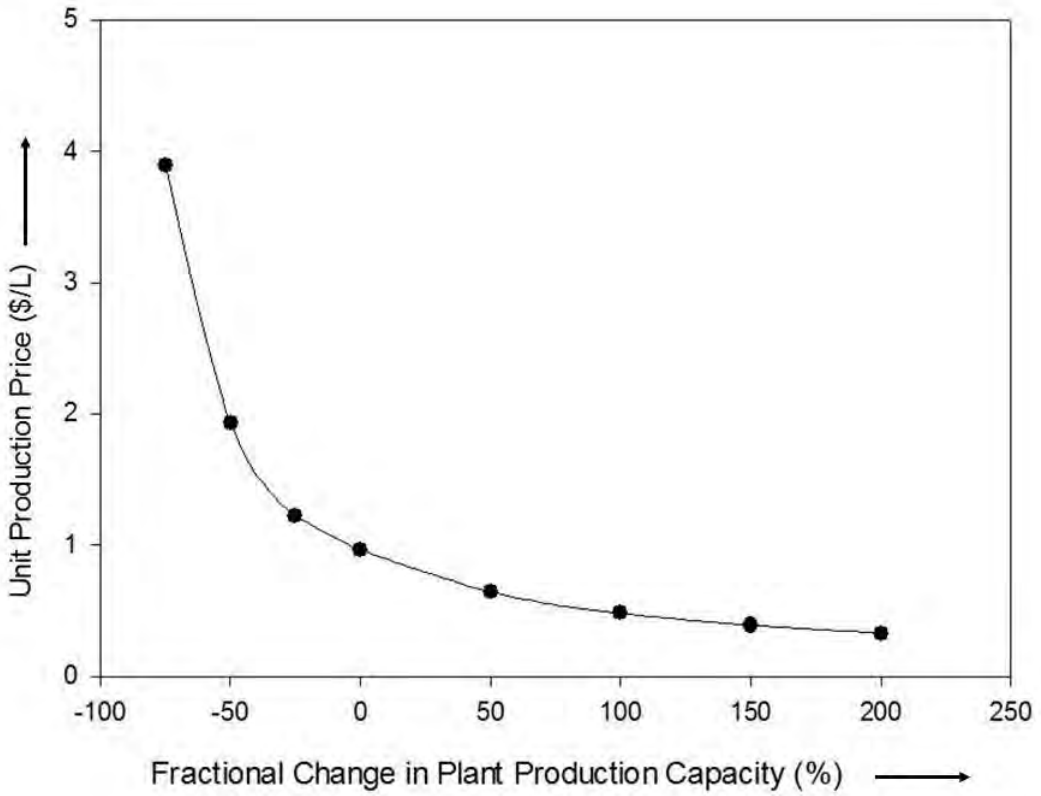

Figure 3. Effect of fractional changes in the plant production capacity on the unit production cost of biohydrogen.

Table 7. Impact of fractional change in annual sugarcane processing capacity on hydrogen production cost

Fractional change (%)	Sugarcane leaves (kg/year)	Hydrogen (L/year)	Production cost ($/L)
-75	14×10^4	331,455	3.89
-50	28×10^4	675,038	1.93
-25	41×10^4	1,026,775	1.28
0	55×10^4	1,384,257	0.96
50	82×10^4	2,110,780	0.64
100	11×10^5	2,847,266	0.48
150	14×10^5	3,590,011	0.39
200	17×10^5	4,336,942	0.33

3.5 Sugarcane leaves as a feedstock

An important factor contributing to the economic profitability of the present plant is the negligible cost associated with the feedstock. Since sugarcane leaves are burnt prior to harvest in South Africa, they are disregarded as a potential feedstock for many bioprocess applications, thus creating a window of opportunity. The cost associated with the feedstock will be the transport costs, as reflected in the annual operating costs. By contrast, other agricultural feedstock may attract a significant portion of the material cost. Qureshi et al. (2013), using wheat straw for butanol production, estimated an annual feedstock cost of >\$3.5 × 10^6 at \$24/ton. Kwiatkowski et al. (2006) reported a feedstock cost of \$31 × 10^6 annually using corn to produce ethanol. Related agricultural wastes that may potentially be used in this plant include rice straw, corn stalk, barley hulls and wheat straw. There have been studies on agricultural feedstocks for hydrogen production, and reported yields are shown in Table 8.

Table 8. Agricultural residues used in biohydrogen production.

Agricultural residue	Hydrogen yield	Reference
Rice straw (RS)	24.8 ml H_2/ g RS	Chen et al. 2012
Corn stalk (CS)	89.3 ml H_2 /g CS	Zhao et al. 2013
Barley hulls (BH)	29.2 ml H_2/ g BH	Magnusson et al. 2008
Wheat straw (WS)	44.68 ml H_2 /g WS	Ivanova et al. 2009

3.6 Using mixed culture from sewage sludge as inoculum in the fermentation unit

The choice of inoculum source has an impact on the annual material costs, thus, a low cost source should be considered. In this simulation, a mixed microbial consortium (anaerobic sludge) was used. Typically, biohydrogen is produced under anaerobic conditions by strictly or facultative anaerobic bacteria. Dark fermentation usually involves hydrolysis and acidogenesis and is the first stage in anaerobic digestion. The estimated theoretical hydrogen yield during the biological conversion of glucose to acetate is illustrated in Equation 5 (Ntaikou et al. 2010).

$$C_6H_{12}O_6 + 4H_2O \longrightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2 \quad (5)$$

In some instances, butyrate is the main organic byproduct, thus the maximum theoretical hydrogen yield decreases to 2 H_2 per mol of glucose, as shown in Equation 6.

$$C_6H_{12}O_6 + 4H_2O \longrightarrow CH_3CH_2CH_2COO- + 2HCO_3^- + 3H^+ + 2H_2 \quad (6)$$

Mixed cultures are often preferred over pure cultures since they reduce asepsis costs and also have higher hydrolysis rates (Ghimire et al. 2015). Mixed cultures have been reported to produce a higher hydrogen yield compared to pure cultures when using lignocellulosic feedstocks. Anaerobic sludge containing a mixed microbial culture produced 63% more hydrogen compared to pure culture *Thermoanaerobacterium* W16 (Table 9). The use of mixed cultures offers the advantage of a negligible cost, thus reducing the material cost. Meyer et al. (2013) reported a cost of 140.00 ¢/lb for a plant using a pure culture of *Saccharomyces cerevisiae* for ethanol production. In a large capacity plant, this cost will make up a significant fraction of the material costs, contributing to the annual operating costs.

Other potential sources of inoculum for this plant include animal droppings, such as from elephant and cow, which have reported a cumulative hydrogen volume and a hydrogen yield of 1300 ml and 68.1 ml H_2/ g VS, respectively (Fangkum and Reungsang 2010, Fan et al. 2006). The cost associated with these inoculum sources would also be minimal.

Table 9. Hydrogen yield from different inoculum sources using lignocellulosic residues.

Inoculum source	Substrate	H_2 yield	Reference
Thermoanaerobacterium W16	Corn stalks	89.3 ml/g CS	Zhao et al. 2013
Activated sludge	Corn stalks	126.22 ml/g CS	Wang et al. 2010
Anaerobic sludge	Corn stalks	141.29 ml/g CS	Ma et al. 2011
Anaerobic sludge	Rice slurry	346 ml H_2/ g VS	Fang et al. 2006
C. thermocellum	Distillers grain	29.2 ml H_2/ g DG	Magnusson et al. 2008

3.7 Possible additional revenue stream

The effluent from this process could be redirected to anaerobic digestion in order to promote methanogenesis and generate methane biofuel. The effluent contains high levels of volatile fatty acids (VFA) namely butyric, acetic and propionic acid. Typically, from glucose, total VFA concentration is approximately 8500 mg/L (Giordano et al. 2011). Methanogenic archaea such as *Methanosarcina* spp. and *Methanosaeta* spp. can anaerobically transform these VFAs into methane (Okudoh et al. 2014). With current natural gas costs approaching $11/1000 cubic feet (EIA 2015), this additional stream of revenue would further increase the overall gross margin of the plant.

4. Conclusion

This study provided a techno-economic analysis at the early pre-commercial stage of biohydrogen production from waste sugarcane leaves within the context of the technology innovation chain. In the current base case plant, a total of 1.4 $x10^6$ L hydrogen is produced annually with an annual operating cost of \$1.3 $x10^6$, thus a unit production cost as low as \$0.96/L. Sensitivity analysis suggested that increasing the plant capacity directly decreases the unit production cost, thus, an indicator of economies of scale. These findings provide data for strategic R&D investment and early stage knowledge on economic viability of biohydrogen production from waste sugarcane leaves.

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Chapter 6

Conclusions and Recommendations for future work

6.1 Conclusions

This study examined the bioprocess development of hydrogen production from waste sugarcane leaves. Based on these findings, the following conclusions can be drawn:

6.1.1 The HCl-based pretreatment model of 4.90 % (v/v) HCl at 99 ^{o}C for 84 min with a S: L of 47.26 % (w/v) gave maximum xylose and glucose yield of 78g/L and 11.48g/L respectively. The xylose: glucose ratio was (7:1) and did not significantly differ between the various acids considered. These findings showed that sugarcane leaf waste which is usually burnt prior to harvest, contain sufficient fermentable sugar that can be recovered through appropriate HCl-based pretreatment, thus indicating its potential as a low-cost feedstock for bioprocesses.

6.1.2 A hydrogen yield of 12.76 ml H_2 g^{-1} FS was achievable using pretreated WSCL at optimum setpoint conditions of 14.23 g/L, 32.68 % (v/v) and 62.77 hr for substrate concentration, inoculum concentration and HRT respectively. These results highlight the importance of optimizing the key operational parameters for biohydrogen process development.

6.1.3 The feasibility of a large scale biohydrogen production was demonstrated at a semi-pilot scale. A cumulative volume and yield of 3739.95 ml and 321 ml H_2 g^{-1} FS respectively was obtained. A peak hydrogen fraction of 37% was obtained. These data indicates sugarcane leaf waste is a viable feedstock for scale-up hydrogen production processes.

6.1.4 A techno-economic analysis at early pre-commercial stage of biohydrogen production from WSCL was carried out. Annually, 1.4 x10^6 L hydrogen is produced with an annual operating cost of \$1.3 x10^6 thus a unit production cost of \$0.96/L. Sensitivity analysis revealed increasing production capacity directly decreased the unit production cost thereby inferring an economy of scales. The study provided data for strategic research and development investment and early stage knowledge on the viability of biohydrogen production from waste sugarcane leaves at a large scale.

6.2 Recommendations for future work

The following recommendations are made from this study:

6.2.1 High yields of xylose and glucose can be recovered from HCl-pretreated waste sugarcane leaves. This low-cost feedstock can be used for biofuel and biomaterial production thus improving the process economics.

6.2.2 The reduction of inhibitory compounds which result from acidic pretreatment of waste sugarcane leaves, such as furfural-derived compounds, will enhance biohydrogen yield on this substrate.

6.2.3 Further scale up studies on this bioprocess will generate knowledge that could be used to operate a pilot plant process with waste sugarcane leaves.

6.2.4 To further enhance process economics, a two-stage process can be considered. The effluent from the biohydrogen process can be treated by anaerobic digestion for methane production thus reducing toxicity and waste treatment cost.

Appendix

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Optimization of xylose and glucose production from sugarcane leaves (*Saccharum officinarum*) using hybrid pretreatment techniques and assessment for hydrogen generation at semi-pilot scale

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ABSTRACT

Lignocellulosic biomass is a promising bioprocess substrate for the production of biofuels and biomaterials. This study modeled and optimized the production of xylose and glucose from sugarcane leaves subjected to hybrid pretreatment of HCl and moist heat and, it examined the dynamics of hydrogen biofuel production from these substrates at a semi pilot scale. The response surface methodology was used to optimize the hybrid pretreatment within the ranges of 4–10%, 60–100 °C and 60–240 min for HCl concentration, temperature and process time respectively. The coefficients of determination (R^2) of 0.99 and 0.93 were obtained for xylose and glucose models respectively indicating the suitability of the models to navigate the optimization space. Process optimization predicted Xylose and glucose yields of 8.92 g/l and 1.68 g/l on a hybrid pretreatment of 5.28% HCl for 187 min at 94.94 °C. Experimental validation yielded xylose and glucose of 8.6 g/l and 1.78 g/l. Biohydrogen production on these optimized substrates in 13L bioreactor showed a peak hydrogen fraction of 26.73% at the 30th hour, and hydrogen yield of 248.05 ml H_2 g^{-1} of fermentable sugar. These findings demonstrate that sugarcane leaves which are usually burnt during harvesting can be an excellent renewable source of fermentable sugars for the production of biofuels such as hydrogen.

Introduction

Plant lignocellulosic biomass constitutes a potential source of low-cost substrate for the production of biomaterials and bioenergy. Its estimated annual production amounts to 200 billion tons [1]. It is considered as waste and its disposal is usually achieved through burning or dumping in landfill sites [2]. In recent time, the use of lignocellulosic material as bioprocess substrate has gained considerable interest due to its renewable nature [3].

Sugarcane leaves, are abundant lignocellulosic material worldwide. Annually, approximately 20 million tons of sugarcane are produced in South Africa [4], 65 million tons in Thailand [5] and 590 million tons in Brazil [6], illustrating its importance as an economic crop. Sugarcane leaves constitute up to 40% of the harvested biomass [5]. These leaves are often

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burned prior to harvest causing damage to the environment [7]. In fact, sugarcane fires have been reported as the largest source of particulate matter which increases ambient carbon monoxide and ozone concentrations in Brazil [8]. Furthermore during the burning process, polyaromatic hydrocarbons (PAH) are also released into the air, and some of these compounds are carcinogenic or mutagenic [9]. These lignocellulosic materials are primarily composed of lignin (15–20%), hemicellulose (20–35%) and cellulose (35–50%) and therefore could potentially be harnessed as a source of carbon and energy for bioprocess production.

The reliance on fossil fuels as the main energy source has led to the rapid depletion of the world reserves. It is predicted that fossil fuels such as oil, coal and gas will be exhausted in the next 35, 107 and 37 years respectively [10]. Additionally the green house gases emitted during their combustions has resulted in global warming [11]. Renewable alternative sources of energy can be generated using microbial systems, provided an appropriate renewable and biodegradable substrate source is used.

The structural nature of lignocellulosic biomass makes it difficult for microbial degradation, thus a suitable pretreatment is required to release the fermentable sugars [12]. Physical pretreatment techniques, such as milling, extrusion, microwave [13] and chemical pretreatment methods requiring acids or bases [13] have been reported. An advantage of acid treatment is the conversion of xylan in hemicellulose to xylose [14]. There are indications that a combination of pretreatment techniques results in a higher release of fermentable sugars [15–17]. For example, a higher glucose production was obtained when a combination of dilute acid and ionic liquid pretreatment was carried out on sugarcane bagasse [18]. It is postulated that cellulose becomes more accessible and hemicellulose solubilizes into monomeric sugars and soluble oligomers when acid and heat pretreatments are combined [14,19].

The Response Surface Methods (RSM) is a modeling and optimization technique which evaluates the interactions of input variables on the process output. It has been applied in modeling and optimization of various fermentation processes [20,16]. To the best of our knowledge the use of RSM in optimization of hybrid treatment of temperature and HCl concentration for optimal production of glucose and xylose from sugarcane leaves has not been reported. Additionally studies on the dynamics of fermentative biohydrogen production on pretreated lignocellulosic substrates at semi pilot scale are scantily reported [21].

This study modeled and optimized the production xylose and glucose from sugarcane leaves using a hybrid pretreatment technique which includes moist heat, treatment time and HCl concentration. The interactive effects of these input parameters on the yield of sugars are examined. Furthermore, an initial assessment of the use of these fermentable sugars for fermentative biohydrogen production at semi pilot scale was investigated.

Materials and methods

Lignocellulosic material

The sugarcane leaves used in this study were collected from The South African Sugarcane Research Institute- SASRI located on the North Coast of South Africa (29°42′18″S, 31°02′44″E) at an altitude of 96 m. This area is characterized by a warm climate with summer rainfall and has an annual mean rainfall of 951 mm. The sugarcane are grown in a fernwood soil which has a mean pH of 7.62 and potassium, calcium and magnesium concentrations of 62.56 mg/l, 1960.79 mg/l and 19.67 mg/l respectively, with clay composing 5% of the soil. Mineral concentrations were determined using the Multi-extractant Ambic-2 method as described by Van der Merwe et al. [22]. Eight months old sugarcane leaves were cut roughly at the 3rd – 6th leaves. They were transported to the laboratory in sealed plastic bags and were dried at 60 °C for 72 h, then reduced to particle sizes of 1 mm using a centrifugal miller (Retsch ZM-1, South Africa). Prior to use, the milled leaves were stored in sealed paper bags.

Experimental design

The Box-Behnken response surface design was used to generate seventeen experimental runs with varied inputs of HCl concentration, heating temperature and heating time of 4–10%, 60 °C–100 °C and 60–240 min respectively (Table 2).

Pretreatment process

5 g of powdered sugarcane leaves were transferred into 250 ml Schott bottles, then 100 ml of HCl at varied concentrations as specified in Table 2 (4.0, 7.0, 10.0% (v/v)) were added into these bottles. Heating was achieved by placing these substrates into a PolyScience Analogue water bath. The holding temperature and heating time set points were maintained as specified in the experimental design (Table 2). The pretreated samples were analyzed for fermentable sugar contents after adjusting the pH to 6.5 with 1 M NaOH. The composition of the untreated and pretreated material was analyzed in duplicates using the procedure described by Goering and van Soest [23]. The content of cellulose, hemicellulose and lignin was further calculated according to Wolfrum et al. [24].

Analytical methods

The xylose and glucose released from each experimental hybrid pretreatment was quantified using the Biochemistry Analyzer (Model 2700 select-dual configuration, YSI USA). The enzyme coupled reactions and electrochemical detection allowed sugar quantification. Glucose was oxidized by glucose oxidase and pyranose oxidase (Eqs. (1) and (2)) while xylose was oxidized by the pyranose oxidase (Eqs. (3)–(5)) which produced hydrogen peroxide (H_2O_2). The H_2O_2 moved through a cellulose acetate membrane where it was oxidized at a platinum electrode. This oxidation produced electrons proportional to the H_2O_2 concentration which correlated with the sugar concentration. Glucose and xylose within a range of 0–9 g/l and 0.5–30 g/l respectively could be quantified.

Prior to usage, the device was calibrated with a 20 g/l xylose and 2.5 g/l glucose calibrator solutions.

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} H_2O_2 + \text{Byproduct} \quad (1)$$

$$H_2O_2 + O_2 \xrightarrow{\text{Pt electrode}} O_2 + 2H^+ + 2e \quad (2)$$

A directly proportionally relationship is observed between glucose and the electron flow.

$$\text{Xylose} + O_2 \xrightarrow{\text{Pyranose oxidase}} H_2O_2 + \text{Byproduct} \quad (3)$$

$$\text{Glucose} + O_2 \xrightarrow{\text{Pyranose oxidase}} H_2O_2 + \text{Byproduct} \quad (4)$$

$$H_2O_2 + O_2 \xrightarrow{\text{Pt electrode}} O_2 + 2H^+ + 2e \quad (5)$$

Scanning electron microscopy (SEM) and phase contrast analysis of sugarcane leaves

The sugarcane leaves (untreated and unmilled, optimally pretreated and unmilled and optimally pretreated and milled) were examined under Scanning Electron Microscopy (SEM). Samples were air dried for 24 h, then mounted on aluminum specimen mounts, gold sputter coated (Eiko IB-3 Ion Coater) and examined using conventional SEM (ZEISS EVO LS 15). Wet mounts were prepared from fermentation effluents and observed via phase contrast microscopy (Olympus BH2).

Model development and validation

The obtained data on xylose and glucose yields from the experimental pretreatments were used to fit the polynomial model equations relating the yields of these fermentable sugars to the pretreatment variables of HCl concentration, heating temperature and heating time. The general form of the model is shown in Eq. (6).

$$Y = \alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_3 x_3 + \alpha_{11} x_1^2 + \alpha_{22} x_2^2 + \alpha_{33} x_3^2 + \alpha_{12} x_1 x_2 + \alpha_{13} x_1 x_3 + \alpha_{23} x_2 x_3 \quad (6)$$

Y represents the response output, α_0 is the intercept, $\alpha_1 x_1$ to $\alpha_3 x_3$ are the linear coefficients, $\alpha_{11} x_1^2$ to $\alpha_{33} x_3^2$ are the quadratic coefficients and $\alpha_{12} x_1 x_2$ to $\alpha_{23} x_2 x_3$ represents the interaction of coefficients. This model was evaluated by Analysis of variance (ANOVA) using Design Expert software (Stat Ease, Inc.). The optimum hybrid pretreatment set points for Xylose and glucose yield were obtained by solving the equations using the methods of Myers and Montgomery [25]. These set points were validated experimentally in duplicate.

Preliminary assessment of pretreated sugarcane leaves for biohydrogen production

Seed inoculum

The Anaerobic sludge from the Darville Wastewater treatment plant (Pietermaritzburg, South Africa), was used as the source of inoculum in this study. The sludge was treated at 121 °C for 10 min to deactivate the hydrogen consuming microorganisms while preserving spore forming microorganisms such as the hydrogen producers. Faloye et al. [26], reported a higher yield of hydrogen (1.35 mol H_2/mol glucose) when inocula was treated at 121 °C compared to 89 °C, which yielded 0.78 mol H_2/mol glucose. Several other studies have found that heat shock treatment results in higher hydrogen yields [27–29].

Semi pilot batch experimental set up

Prior to the semi pilot scale process, an intermediate seed stage was carried out in a 1000 ml fermentation vessel. The vessel was fed with 175 ml of pretreated sugarcane leaves (pH adjusted to 6.5 with 1 M NaOH) and 175 ml of mineral salts. The mineral salt mix was made up of the following in g/l: NH_4Cl 0.5, KH_2PO_4 0.5, K_2HPO_4 0.5, $NaHCO_3$ 4.0, $FeCl.2H_2O$ 0.15, $MgCl.6H_2O$ 0.085, $ZnSO_4.7H_2O$ 0.01, $MnCl_2{\cdot}4H_2O$ 0.03, H_3BO_3 0.03, $CaCl_2.6H_2O$ 0.01, $Na_2MoO_2{\cdot}2H_2O$ 0.03. This substrate was inoculated with 150 ml of treated sludge. The pH of all contents in the flask was adjusted to 6.5 with 1 M NaOH. The culture was flushed with nitrogen gas for 2 min, and incubated at 37 °C in a shaking water bath at 100 rpm. This intermediate stage was monitored for an exponential phase of biohydrogen production.

The semi pilot scale fermentation was carried out in a 10L bioreactor (Labfors-INFORS HT, Switzerland). The bioreactor was sterilized, fed with 2250 ml of pretreated sugarcane leaves and 2250 ml of the defined mineral salts. It was then inoculated with 500 ml of the seed culture from the intermediate stage. The vessel was flushed with nitrogen gas for 3 min prior to fermentation in order to create an anoxic environment. The control set points of pH, temperature and agitation rate were maintained at 6.15, 37 °C and 250 rpm respectively for 68 h.

Process monitoring

The evolving biogas from the fermentation process was continuously monitored through an array of three sensors to determine the fraction of hydrogen, methane and carbon dioxide in realtime. The biogas sensors used were (BCP–H_2), (BCP–CH_4) and (BCP–CO_2) with measuring ranges of (0–100 vol%), (0–100 vol%) and (0–50 vol%) respectively (Bluesens, Germany). The biogas volume was monitored using a milligas counter (MGC, Bluesens, Germany). A sampling interval of 1 min was adopted. The broth samples were collected every 6 h and analyzed for xylose and glucose consumption using the Biochemistry Analyzer.

Results and discussion

Composition of sugarcane leaves

The analysis of raw sugarcane leaves indicated that the hemicellulose, cellulose and lignin components were 28.28%, 44% and 10.04% respectively (Table 1) correlating with previous reports on sugarcane leaves composition [30,31]. The optimized hybrid pretreatment showed a solubilization of hemicellulose and cellulose of 4.14% and 18.96% respectively. Surprisingly, the content of lignin increased by 0.98%. This slight increase in lignin content could be accounted by the

Table 1 – Chemical composition of raw and pretreated Sugarcane leaves.

Sample	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Raw	44	28.28	10.04
Treated	25.04	24.14	11.02

pseudo-lignin formation which has previously been reported [32,33]. The decrease in hemicellulose and cellulose content occurred as the acid pretreatment hydrolyzed the polysaccharides into monosaccharides [12]. The high solubilization of cellulose and subsequent low yield of glucose could be attributed to the acid catalyzed dehydration of the sugar intermediates into furfural-type components [34].

Modeling of xylose and glucose release on the hybrid pretreatment

The experimental data from the hybrid pretreatment conditions were used to develop two polynomial model Equations relating xylose and glucose yields to HCl concentration, heating temperature and heating time (Eqs. (7) and (8)). The fitness of the models was assessed using Analysis of Variance (ANOVA). The result is presented in Table 3. The coefficients of determination (R^2) for xylose and glucose models were 0.99 and 0.93 respectively, indicating that these models could account for 99% and 93% of variations in the observed data. The relatively low p-values of <0.0001 and 0.0019 and the high F values of 101.48 and 11.71 further elucidate the significance of these polynomial models (see Table 4).

The final equations modeled in terms of coded factors were:

$$\text{Xylose (g/l)} = 9.14 + 0.99A + 0.49B + 2.07C - 0.14AB - 2.12AC - 1.50BC - 0.050A^2 + 7.275E\text{-}003B^2 - 3.45C^2 \quad (7)$$

$$\text{Glucose (g/l)} = 1.21 - 0.43A + 0.14B + 0.45C - 0.12AB - 0.24AC + 0.012BC - 0.67A^2 - 0.14B^2 - 0.084C^2 \quad (8)$$

Linear effect of pretreatment variables on the xylose and glucose yields

The yield of xylose and glucose ranged from 1.275 g/l to 10.281 g/l and from 0 g/l to 1.81 g/l respectively indicating the sensitivity of fermentable sugar release to the considered input variables.

Table 3 – Analysis of Variance (ANOVA) for xylose and glucose models.

Source	Sum of squares	df	Mean squares	F-value	P-value	R^2
Xylose Model	121.83	9	13.54	101.48	<0.0001	0.99
Glucose Model	5.61	9	0.62	11.71	0.0019	0.93

df: degrees of freedom, F-value: Fisher-Snedecor distribution value, P-value: probability value, R^2: coefficient of determination.

HCl concentration

As shown in Table 2, the hybrid pretreatment carried out at high HCl concentration (10%) showed very low yield of glucose (0 g/l) and high yield of xylose (10.08 g/l) whereas treatments at low acid concentration (4%) gave a relatively low yields of both xylose and glucose (<1 g/l) (Figs. 4 and 5). A similar effect of acid concentration on sugar yield from cellulosic plant biomass has been reported by Sindhu et al. [16] and Saha et al. [35]. These authors observed an increase in glucose yield from 15 mg/g to 20 mg/g and an increase in xylose plus galactose from 0 mg/g to 158 mg/g when the acid concentration was increased from 0% to 1.00% (w/v). At high concentrations of HCl (>10%) the glucose and xylose yields were 0 g/l and 6.384 g/l respectively, while at a lower HCl concentration (4%), both xylose and glucose had relatively high yields (8.826 g/l and 1.398 g/l respectively).

Temperature and time

Hybrid pretreatment carried out at low temperature (60 °C) gave a very low yield of glucose and xylose (0.664 g/l and 0.455 g/l respectively) compare to pretreatment at higher temperature (100 °C) which gave xylose and glucose yields of 8.826 g/l and 1.398 g/l respectively. This trend has been reported in the solubilization of glucan and xylan from cotton stalks [36].

Table 2 – Box-Behnken design used for hybrid pretreatments of Sugarcane leaves on variables of HCl concentration, heating time and temperature.

Run	A: HCl (%)	B: Heat duration (Mins)	C: Heat temperature (°C)	Response 1 xylose (g/l)	Response 2 glucose (g/l)
1	10.00	60.00	80.00	10.084	0
2	7.00	150.00	80.00	9.527	1.116
3	7.00	240.00	100.00	7.112	1.81
4	4.00	60.00	80.00	7.632	0.569
5	10.00	240.00	80.00	10.281	0
6	10.00	150.00	100.00	6.385	0
7	7.00	60.00	100.00	8.618	1.482
8	7.00	240.00	60.00	5.763	0.47
9	4.00	150.00	60.00	0.644	0.445
10	7.00	150.00	80.00	8.969	1.233
11	7.00	150.00	80.00	8.905	1.213
12	7.00	150.00	80.00	9.278	1.271
13	10.00	150.00	60.00	6.684	0
14	4.00	240.00	80.00	8.389	1.058
15	7.00	60.00	60.00	1.275	0.191
16	4.00	150.00	100.00	8.826	1.398
17	7.00	150.00	80.00	9.017	1.223

Table 4 – Models coefficient of estimates with standard errors.

Factor	Xylose coefficient estimate	Xylose standard error	Glucose coefficient estimate	Glucose standard error
Intercept	9.14	0.16	1.21	0.10
A	0.99	0.13	−0.43	0.082
B	0.49	0.13	0.14	0.082
C	2.07	0.13	0.45	0.082
AB	−0.14	0.18	−0.12	0.12
AC	−2.12	0.18	−0.24	0.12
BC	−1.50	0.18	0.012	0.12
A^2	−0.050	0.18	−0.67	0.11
B^2	7.275E-003	0.18	−0.14	0.11
C^2	−3.45	0.18	−0.084	0.11

Similarly, a low heating time (60 min) resulted in a lower yield of xylose and glucose (7.632 g/l and 0.569 g/l respectively) while pretreatment with higher heating time showed an increase in the xylose and glucose yield (8.389 g/l and 1.058 g/l respectively).

Interaction of experimental variables on glucose and xylose yields

The interactive effect of the hybrid pretreatment parameters was examined using the response surface graphs. It was observed that acid concentration and heating time have a linear relationship on xylose yield (Fig. 1). When these parameters were varied from their lower to higher levels, so did the xylose concentration increase from 0 g/l to 10.2 g/l.

As shown in Fig. 2, if the temperature was maintained at 92 °C and HCl concentration was increased from 4 to 10%, the xylose concentration also increased from 7.5 g/l to 10 g/l. Similarly as shown in Fig. 3, if the heating temperature was maintained at 92 °C, an increase in heating time from 60 min to 240 min resulted in an increase in xylose yield from 7.5 g/l to 9.8 g/l.

The interactive effect of heating time and HCl concentration when the heating temperature was kept at its median value is shown in Fig. 4. Higher glucose yields (>1 g/l) could be achieved using lower HCl concentration (<6%) while maintaining a higher treatment time (240 min). Similarly, the glucose yield peaked at 1.7 g/l at a low acid concentration (5%) with a higher heating temperature (100 °C), as shown in Fig. 5. These observations indicate that a higher glucose yield can be achieved at a low acid concentration (5%) with high heating time (240 min). A decreasing trend in glucose yield was observed when the HCl concentration was increased from 6 to 10%. Similar trends have been reported by Dussan et al. [37] where the yield of glucose decreased significantly as the acid concentration increased in the pretreatment of sugarcane bagasse. Fig. 6 shows the effects of heating temperature and heating time on glucose yield. Pretreatment temperature below 80 °C applied for durations below 150 min gave low glucose yield from sugarcane leaves.

SEM analysis

As observed in the electron micrographs, the optimal hybrid pretreatment degraded the surface of the leaves producing a rough layer with exposed inner materials (Fig. 7) compared to untreated leaf samples which have a relatively smooth surface and showed fibers and a mosaic of binding materials (Fig. 8). In contrast, Fig. 9 showed the unraveling of the

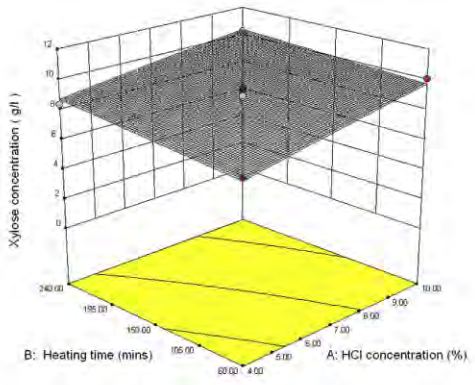

Fig. 1 – 3-D response surface plot showing the interaction of heating time and HCl concentration on xylose yield.

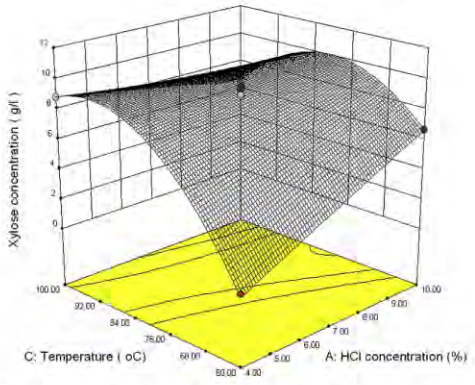

Fig. 2 – 3-D response surface plot showing the interaction of heating temperature and HCl concentration on xylose yield.

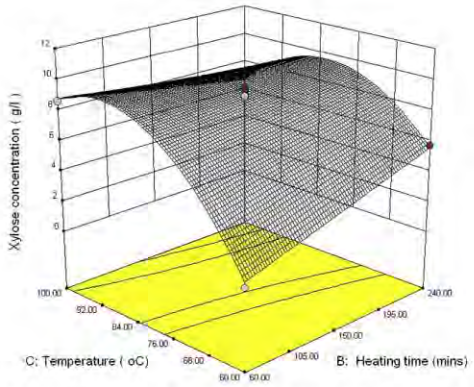

Fig. 3 – 3-D response surface plot showing the interaction of heating temperature and heating time on xylose yield.

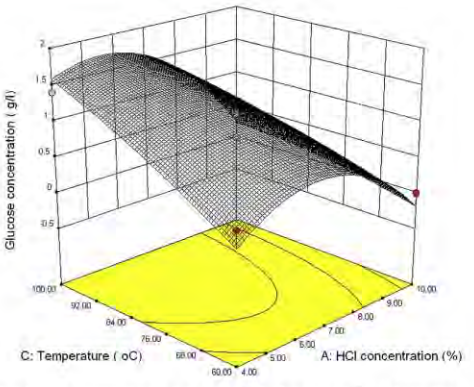

Fig. 5 – 3-D response surface plot showing the interaction of heating temperature and HCl concentration on glucose yield.

epidermal layer after acid and heat treatment. Major structural damages were observed in Fig. 7 where the sample underwent milling which altered the cellulose crystallinity. This process greatly enhanced the acid solubilization of the inner structures of the leaves.

Optimization of sugarcane leaves pretreamtent on xylose and glucose production using a hydrid technique

The optimized hybrid pretreatment conditions based on the developed process model predicted xylose and glucose production of 8.92 g/l and 1.68 g/l respectively using optimal set points of HCl concentration, heating temperature and heating time of 5.28%, 94.40 °C and 187 min respectively (Table 5). Experimental validation carried out in duplicates gave average xylose and glucose yields of 8.6 g/l and 1.78 g/l respectively (Table 5).

Preliminary assessment of biohydrogen production from semi pilot batch system

During the semi pilot batch process, the hydrogen production started at the 30th hour of fermentation and reached its exponential course at the 36th hour. Cells showed a preferential affinity for glucose metabolism at the early stage of the fermentation. During the lag phase the glucose concentration decreased from 2.096 g/l to 0.9 g/l, while the xylose concentration remained relatively stable. As the initial concentration of glucose was relatively low, there was no observable increase in hydrogen production during this time. Presumably, glucose was channeled for cells adaptative metabolism, and the lengthy lag phase may be accounted by the low concentration of glucose. Xylose metabolism started about 27 h into the process. The exponential phase of

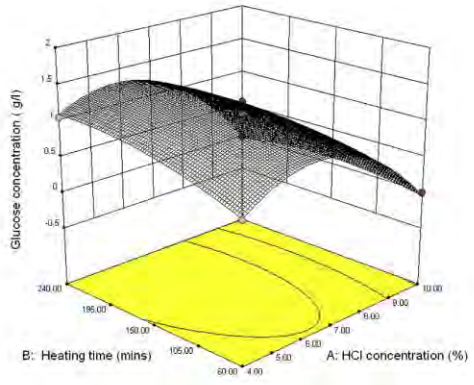

Fig. 4 – 3-D response surface plot showing the interaction of heating time and HCl concentration on glucose yield.

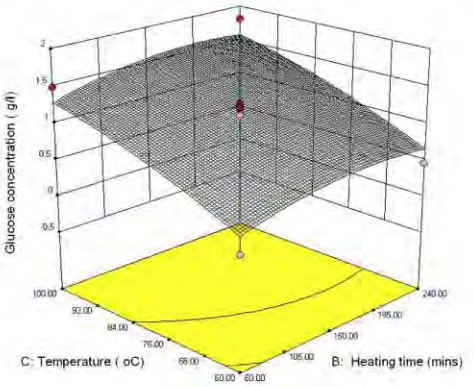

Fig. 6 – 3-D response surface plot showing the interaction of heating temperature and heating time on glucose yield.

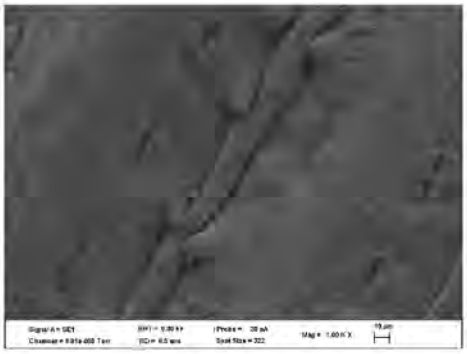

Fig. 7 – Scanning electron micrograph of the optimally pretreated sugarcane leaf.

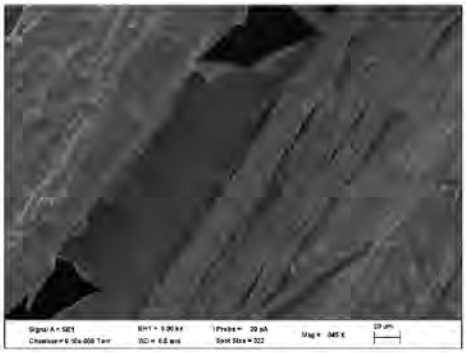

Fig. 9 – Scanning electron micrograph of the optimally pretreated sugarcane leaf (unmilled).

hydrogen production was initiated at the 36th hour and lasted for 29 h leading to a peak hydrogen fraction of 26.73%. Lin et al. [38] reported an exponential phase lasting 25 h using xylose as a substrate for hydrogen production in batch experiments. As shown in Fig. 10, a steady fraction of hydrogen concentration at a peak value of 26.73% was maintained for approximately 1 h, followed by a decline in the hydrogen fraction. This decline was presumably caused by the depletion of xylose (0.2 g/l–0 g/l). The carbon dioxide production showed a steady increase from 30 h to 37 h with a peak fraction of 38.07% and a cumulative volume of 3183.23 ml. This increase in CO_2 is most likely a result of the acetic acid metabolic pathway being favored, which generated 3 mol CO_2 mol^{-1} xylose. It has been suggested that the hydrogen partial pressure could affect the amount of CO_2 released during hydrogen production [39].

Microorganisms utilizing xylose require a shift in metabolic pathway in which either the phosphoketolase pathway or the pentose phosphate pathway is used [40]. The hydrogen fraction of the evolving biogas increased exponentially from 0.842% to a peak value of 26.73% at the 65th hour. A corresponding decrease in xylose concentration from 6 g/l down to 0.2 g/l was observed during this period (Fig. 10). The fermentation process gave a cumulative hydrogen production of 2117.91 ml. Significant improvement on the hydrogen yield could be achieved by optimizing the operational parameters such as pH, temperature, agitation rate, hydraulic retention time and organic loading rate.

Phase contrast microscopy of the fermentation process effluent showed rod and club-shaped organisms (Fig. 11), indicating the presence of presumptive species of Clostridia. These organisms have been reported as the main hydrogen producers when treated sludge is the primary inoculum [39,41]. The presence of endospores is further supported by the lack of fermentable sugars in the effluent, creating unfavorable conditions for the spore-forming microorganisms [42].

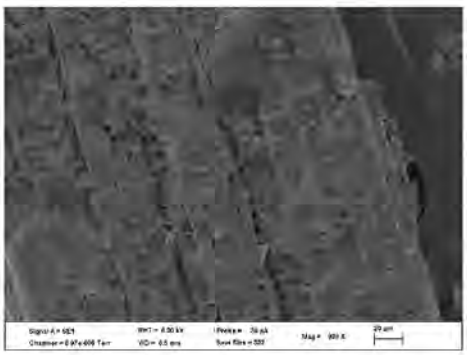

Fig. 8 – Scanning electron micrograph of the untreated sugarcane leaf.

Conclusion

The bioprocess models relating the release pattern of xylose and glucose from sugarcane leaves subjected to hybrid pretreatment of HCl and moist heat have been developed. The optimized hybrid pretreatment produced xylose and glucose

Table 5 – Optimum levels of variables during pretreatment of Sugarcane leaves.

Independent variables		Predicted optimum levels
HCl concentration		5.28%
Heating time		187 min
Temperature		94.94 °C
Response	Prediced value	Observed value
Xylose	8.92 g/l	8.6 g/l
Glucose	1.68 g/l	1.78 g/l

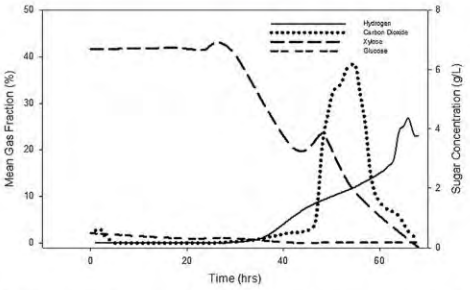

Fig. 10 – Time course of hydrogen production and sugar consumption rate for the pilot scale-up process.

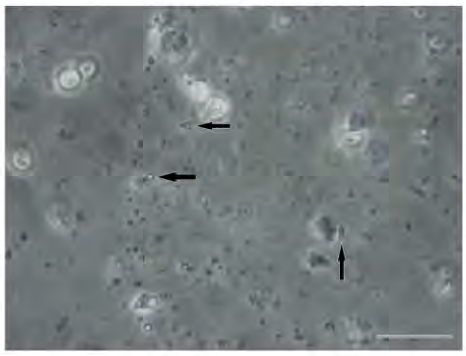

Fig. 11 – Phase contrast microscopy showing the presence of endospore formers.

concentrations of 8.6 g/l and 1.78 g/l respectively. Using these sugars for a semi pilot scale fermentative biohydrogen production, a prioritized microbial metabolism of glucose over xylose and an extended lag phase of 30 h were observed. A peak hydrogen fraction of 26.73% and a hydrogen yield of 248.05 ml H_2 g^{-1} were obtained. It is anticipated that the determination of optimum physico-chemical set points of substrate concentration, hydraulic retention time and inoculum concentration could greatly impact the hydrogen output on this substrate. An additional interest lies in the renewability, abundance and the challenge in disposal of this substrate which inherently would positively impact the process economics.

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