

**OPTIMIZATION OF XYLOSE AND GLUCOSE YIELDS FROM NAPIER
GRASS (*Pennisetum purpureum*) USING HYBRID PRETREATMENT AND
ASSESSMENT FOR FERMENTATIVE HYDROGEN PRODUCTION
USING IMMOBILIZED CELLS**

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

Master of Science

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Optimization of xylose and glucose yields from napier grass (*Pennisetum purpureum*) using hybrid pretreatment and assessment for fermentative hydrogen production using immobilized cells

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

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DECLARATION 2 - MANUSCRIPTS SUBMITTED FOR PUBLICATION

Publications forming part of this thesis include published and submitted manuscripts. The first author (student) contributed in experimental work, data analysis and manuscript preparation, guided by the second author (supervisor).

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Mafuleka S, Gueguim Kana EB. (2015). Modelling and optimization of xylose and glucose production from napier grass using hybrid pretreatment techniques. *Biomass & Bioenergy* 77, 200 – 208.

Manuscript 1

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ABSTRACT

The dependence on non-renewable fossil fuels has led to the depletion of these energies and global warming. Thus there is a need to search for sustainable energy sources to mitigate these challenges. Lignocellulosic biomass is an excellent potential substrate for renewable biofuel production due to its high carbohydrate content, abundance and sustainability. The polymer interlinks of cellulose, hemicellulose and lignin hinder effective hydrolysis of biomass for the production of fermentable sugars. Efficient and cost effective pretreatment is required for utilization of lignocellulosic materials as substrates for biofuel and biomaterial.

In this study, four hybrid techniques of napier grass pretreatment, namely HCl and moist heat (HH), HCl and microwave (HM), NaOH and moist heat (NH) and NaOH and microwave (NM) were modeled and optimized for xylose and glucose production using Response Surface Methodology (RSM). The optimized pretreatment conditions of HH gave 12.83 g/l xylose and 2.28 g/l glucose, and optimized HM pretreatment gave 15.06 g/l and 2.44 g/l xylose and glucose respectively. A xylose to glucose ratio of 5.6:1 was obtained for the optimized HH pretreatment compared to 6.1:1 for the optimized HM pretreatment. For NH and NM hybrid pretreatments, low concentrations of fermentable sugars were observed. The coefficients of determination (R^2) of 0.83 and 0.97 were obtained for xylose and glucose production respectively using HH hybrid pretreatment, and 0.90 and 0.80 were obtained for xylose and glucose respectively using HM hybrid pretreatment. The optimum generation of xylose and glucose from napier grass indicates its potential as substrate for the production of renewable biofuels.

Furthermore, the optimum physico-chemical set points of Hydraulic Retention Time (HRT) and substrate concentration were investigated for hydrogen production on napier grass using immobilized beads. The optimized set points were 139.97 hours and 19.05% for HRT and substrate concentration respectively, with predicted yield of 5.31ml H₂/g napier grass. Model validation gave 6.61ml H₂/g napier grass. To assess the dynamics of hydrogen generation at semi-pilot scale, biohydrogen production was carried out in a 13L bioreactor. A peak hydrogen fraction of 28.52% and hydrogen yield of 14.03ml H₂/g napier grass was observed at pH 6.3, temperature 37°C at 62 hours. This optimum generation of biohydrogen using renewable napier grass highlights potential application of this feedstock for large scale biofuel production. Additionally, dark fermentative hydrogen production from napier grass using immobilized

microbial consortia combined a cheap hydrogen production method with high unit volumetric production rate thus positively impacting the bioprocess economics.

Keywords: Biohydrogen production, Napier grass, Lignocellulosic biomass, Modeling, Optimization, Dark fermentation, Biofuels

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
CH ₄	methane
CO ₂	carbon dioxide
DEA	Department of Environmental Affairs
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
H ₂	hydrogen
HRT	Hydraulic Retention Time
IEA	International Energy Agency
IPCC	Intergovernmental Panel on Climate Change
PCR	Polymerase Chain Reaction
rpm	revolutions per minute
RSM	Response Surface Methodology
VFA	Volatile Fatty Acids
W	Watts
WHO	World Health Organization

CHAPTER 1

General introduction

1.1 Fossil fuels depletion and their negative impacts

The excessive use of the exhaustible fossil fuels has led to its hasten depletion, abrupt climate fluctuations and soaring concerns over environmental deterioration (Azwar *et al.*, 2014). The global estimates of proven fossil fuel reserves and consumption through the end of 2009 as appraised by international agencies and energy corporations is shown in figure 1.1. Fossil fuels have been the driving force for industrial revolution and economic growth. About 80% of the world's primary energy is fossil fuel derived with oil, coal and natural gas accounting for 32.8, 27.2 and 20.9% respectively (IEA, 2011). Annual production of fossil fuel derived energy has grown from 1800 to about 10 000 million tons per year from the year 1800 to 2010 (Hook and Tang, 2013). Projections show that conventional oil is fast approaching peak production. With approximately 1100Bboe (billion barrels of oil equivalent) consumed from the year 1800 to 2009 which is almost half the proven oil reserves of approximately 2500Bboe (Figure 1.1). Conventional oil consumption and demand is expected to increase with increasing population growth and economic development (Leggett and Ball, 2012). Bentley *et al.* (2007) predicts peak conventional oil production before 2030. These observations indicate a looming energy crisis. The worlds proven coal reserves are judged sufficient to meet the projected growth for the next 107 years (Shafiee and Topal, 2009). However, its processing and consumption has brought adverse environmental impacts. These include significant greenhouse gas emissions and acid mine drainage (McCarthy and Pretorius, 2009). South Africa is the 15th largest CO₂ emitter at global scale from coal combustion (DEAT, 2009). The global average CO₂ emissions through coal combustion are 4.28 ton CO₂ per annum while that of South Africa is 7.22 ton CO₂ per annum (IEA, 2008).

CO₂ is the primary greenhouse gas and the main contributor to the global temperature increase (Sathre, 2014). Figure 1.2 presents the main greenhouse gases emissions through to the end of the year 2007. A significant amount of CO₂ (57%) is emitted from fossil fuel combustion and processing. In 2007, 8365 million metric tons of CO₂ was emitted into the atmosphere with

76.3% from fossil fuel combustion and consumption (Boden *et al.*, 2010). Atmospheric CO₂ concentrations have increased from 280 ppmv in the 18th century to 390 ppmv in 2010 (NRC, 2010). Studies project a continuous increase to as much as 1000 ppmv by the year 2100 (IPCC,2007). This will result in a mean global temperature increase of between 1.5 to 4.5°C which will have profound effects on growth and physiology of plants and mankind (Ziska, 2008). Unless corrective measures are taken, mankind will be adversely affected since extremely high temperatures cause cardiovascular and respiratory diseases (Menne and Ebi, 2006). The extreme temperatures also increase the evaporation rate of surface waters which will cause lack of fresh water, compromising hygiene and increasing diarrheal diseases (Stone and Weaver, 2003). Thus there is significant interest towards the production of non-carbonaceous fuels from renewable feedstocks to alleviate these concerns and to meet the future energy supply (Leggett and Ball, 2012).

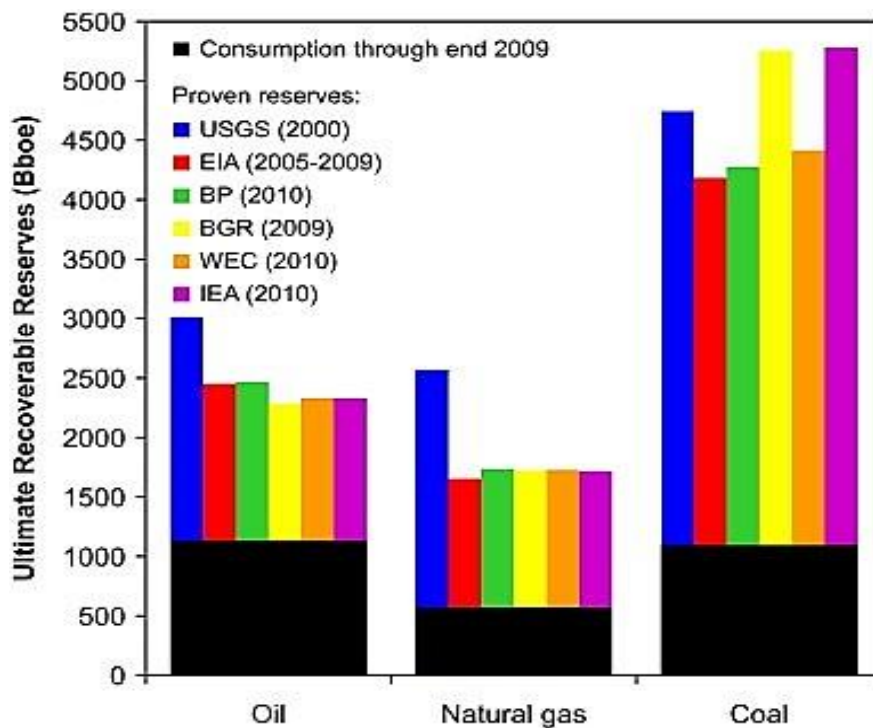


Figure 1.1- The global estimates of proven fossil fuels reserves and consumption as appraised by international agencies (Chaira and Zecca, 2011).

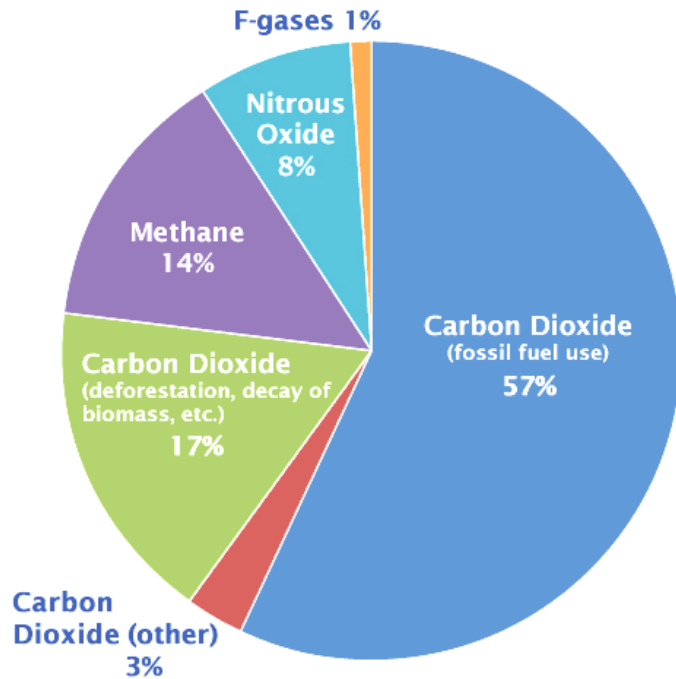


Figure 1.2 - The global scale of main greenhouse gasses emissions (IPCC, 2007)

1.2 Hydrogen as an alternative energy

Hydrogen offers potential as alternative energy currency to fossil fuels energy (Wong *et al.*, 2014). It has high energy yield (141.9kJ/g) and generates only water from its oxidative combustion thus making it the most ideal and eco-friendly alternative to fossil fuels (Midilli *et al.*, 2005; Piera, 2006). Its production and consumption has already commenced globally with an estimated annual growth rate of 3.5% (Freedonia Group, 2010). Hydrogen is currently produced through steam reforming of natural gas, coal carbonization and partial oxidation of heavy oil (Dunn, 2002). Its commercialization as an alternative energy source is still uneconomical due to high production costs (Dunn, 2002). Thus there is an urgent need to develop low cost and environmentally friendly hydrogen production technologies. One promising cost effective technique for commercial hydrogen production is through biological processes. Dark fermentation is a prominent biological approach due to: (a) its use of simple and cheap bioreactor configurations (b) a range of substrates like lignocellulosic biomass substrates, sludge, wastewater and others with no light and oxygen demand (Mohan *et al.*, 2008). Dark fermentation can use diverse mixed microbial consortia providing synergist pathway interactions thus

improving substrate degradation and enhancing biohydrogen production (Kapdan and Kargi, 2006). Major limitations to implementation of fermentative hydrogen production at industrial scale are high production costs and low substrates conversion efficiencies (Li and Fang, 2007). The use of cheap and renewable feedstocks such as lignocellulosic biomass substrates could significantly lower the hydrogen production cost thus making its production economically feasible at industrial scale.

1.3 Potentials of lignocellulosic substrates for biohydrogen production

Lignocellulosic feedstocks are amenable for fermentative hydrogen production since the raw material is abundant and sustainable. The global production of lignocellulosic biomass is estimated above 220 billion tons per annum (Ren *et al.*, 2009). These large quantities of lignocellulosic biomass accumulate from agriculture, forestry and other agro-industries. Disposal of these biomass substrates raises environmental concerns since these are resistant to natural biodegradation by microorganisms and microbial enzymes (Howard *et al.*, 2003). Thus, they remain in landfill sites for years harboring rats, flies, snakes and breeds diseases vector insects. A global phenomenon thus is to dispose lignocellulosic biomass from the environment by burning (Howard *et al.*, 2003). This practice however emits significant amounts of CO₂ into the atmosphere and poses an environmental pollution problem (Levin, 1996). Lignocellulosic feedstock chemical properties make these substrates of enormous biotechnological value as cheap sources of fermentable sugars. Thus, the large quantities of lignocellulosic biomass feedstocks generally considered as “waste” could be converted into valuable biohydrogen, other biofuels and value-added products. Lignocellulosic feedstock selection depends on fermentable sugars content, availability and costs of the substrate (Kapdan and Kargi, 2006). In Africa, napier grass (*Pennisetum purpureum*) holds potential as a competitive feedstock for biohydrogen production. Napier grass is a C₄ all season grass species that is native to and widely distributed in African grass lands. It has high holocellulose content (up to 69%), high biomass yields (about 40 metric ton/ha/year) and is rapid growing. Globally, Somerville *et al.* (2012) reported annual cumulative dry matter napier grass yields of 85 tons per hectare, while Reddy *et al.* (2012) reported an annual cumulative yield of 40 tons per hectare in South Africa. However, the compositional and components interaction of cellulose, hemicellulose and lignin polymers in napier grass limit the effective hydrolysis of biomass for fermentative hydrogen production (Wongwatanapaiboon *et al.*, 2012).

1.4 Challenges associated with the use of lignocellulosic substrates

Lignocellulosic biomass is composed of inter-linked cellulose, hemicellulose and lignin. Cellulose is composed of thousands of β -glycosidic bond linked glucose molecules. Hemicellulose consists of hexoses, pentoses and sugar acids, and it binds to cellulose molecules in biomass. Lignin is made up of a framework of phenyl propane units namely coniferyl, guaracyl, syringyl and syringyl alcohol. High concentrations of these phenyl propane units gives biomass rigidity, protection against pathogen attacks, provides strength, water proof and hindrance to hydrolysis by forming a steric barrier limiting enzymatic penetration (Kumar *et al.*, 2008). Lignocellulosic biomass requires pretreatment or hydrolysis to break the lignin seal, structure and chemical components of the feedstock prior to use for hydrogen fermentation. Lignocellulosic biomass pretreatment allows easy access of microbial enzymes to fermentable cellulose and hemicellulose thus improving substrate enzymatic digestibility. Furthermore, it hydrolyses cellulose and hemicellulose to monomeric sugars predominantly xylose and glucose. Ideally a pretreatment method should be low cost, efficient, have high sugar yields and produce limited or no toxic byproducts such as phenolic compounds (Kapdan and Kargi, 2006).

The mechanical, physico-chemical, physical and chemical pretreatment procedures and combinations have been used to pretreat lignocellulosic biomass substrates. These include steam explosion, alkali and acid hydrolysis, wet oxidation, ammonia fiber explosion, biological and microwave hydrolysis as some examples of biomass pretreatment strategies. The hydrolysis of napier grass through steam explosion has been reported by Chang *et al.* (2011) under varied temperature from 160 to 210°C and a reaction time between 2 to 20 minutes. The authors reported a significant reduction in glucan, xylan and lignin (67.3, 6.83 and 20.02% respectively) at 210°C for 20 minutes. Steam explosion however, results in xylan fraction destruction, toxic and inhibitory phenolic compounds production, incomplete lignin-carbohydrate matrix disruption and has high-energy requirements (Mackie *et al.*, 1985). Biological pretreatment has been used to hydrolyze lignocellulosic biomass (Zhang *et al.*, 2008). This method uses fungal enzymes to degrade lignin, hemicellulose and polyphenols. Industrial application of this method is limited by slow degradation rate and microorganisms consuming some hydrolyzed carbohydrate fraction. Acid pretreatment solubilizes lignin and hydrolyzes hemicellulose to xylose, thus making cellulose accessible to enzymatic hydrolysis (Eggeman and Elander, 2005). This strategy is inexpensive and efficient, however it causes corrosion to the bioreactor internal structures

(Lopez-Arenas *et al.*, 2010). Alkaline pretreatment strategy disrupts the lignin structure of biomass but also removes uronic acid substitutions on hemicellulose thus reducing accessibility of hemicellulose to hydrolytic enzymes (Cardona and Sachnez, 2006). Microwave radiation and thermal pretreatment strategies disrupt lignin, reduce degree of polymerization of biomass and hydrolyze hemicellulose to xylose. These pretreatment strategies have high energy requirements and are slow (require long process times) (Lopez-Arenas *et al.*, 2010). Ammonia fiber explosion uses hot liquid ammonia (<90°C) under high pressure for specific duration (<30 minutes) to delignify and solubilize hemicellulose (Cardona and Sachnez, 2006). This process is highly efficient, however the costs of ammonia and recovery process make this pretreatment economically unviable (Cardona and Sachnez, 2006). Thus, alternative more efficient and cost effective biomass pretreatment approaches are investigated.

Studies have shown that each of the single pretreatment gives limited sugar yields due to specific mode of action (namely HCl only targets hemicellulose primarily, but not lignin) and intrinsic disadvantages. To overcome this, combined or hybridized pretreatment methods using two or more pretreatment techniques on biomass are explored. The pretreatment combinations have been reported to enhance sugar yields, reducing pretreatment duration and increasing severity. However, their use could increase pretreatment costs. Hybrid pretreatment techniques of steam explosion and dilute 1% H₂SO₄ on raw wheat straw have been reported with yield improvement of 8 to 10g glucose/100g raw wheat straw compared to 6.4g glucose/100g wheat straw obtained without acid addition (Lopez-Arenas *et al.*, 2010).

An obstacle to the commercialization of biohydrogen produced from lignocellulosic feedstocks is the low microbial unit production rate in dark fermentation. One approach to improving the microbial unit production rate is through microbial biomass retention within a reactor using cell immobilization processes. Immobilized cell systems allows better biomass retention at high dilution rates, creates a local anaerobic environment and maintains high microbial cell densities compared to suspended cells which increases hydrogen yields (Wu *et al.*, 2003). Cell immobilization uses methods such as adsorption of cells onto solid surfaces, biofilms, granules and entrapment onto synthetic polymers for biomass immobilization (Fang and Liu, 2002; Palazzi *et al.*, 2000). Studies have reported better hydrogen yields from immobilized cells compared to suspended cells using pure glucose and palm oil mill wastewater as substrates (Wu *et al.*, 2003). Fermentative hydrogen production using immobilized mixed microbial consortium on

lignocellulosic feedstocks is scantily reported.

1.5 Problem statement

Due to the looming energy crisis and the soaring concerns over environmental deterioration owing to the anticipated fossil fuel depletion and fossil fuel combustion, a clean and sustainable alternative energy is of urgent demand (Ren *et al.*, 2009). Biohydrogen is emerging as a promising energy carrier to alleviate the reliance on the exhaustible fossil fuels (Ho *et al.*, 2012). However, its high production cost is a major obstacle to commercialization. The use of cheap and renewable feedstocks such as grasses could make hydrogen production economically feasible. Grasses are attractive feedstocks due to their high carbohydrate content, abundance, sustainability and local availability (Wongwatanapaiboon *et al.*, 2012). Napier grass is a promising feedstock for biohydrogen production because of its high cellulose content, rapid growth, highly invasive nature, high adaptability and high biomass yields. Globally, annual cumulative dry matter napier grass yields of 85 tons per hectare have been reported (Somerville *et al.*, 2012), with 40 tons per hectare in South Africa (Reddy *et al.*, 2012). These large quantities of napier grass accumulated yearly are disposed of by burning since the biomass is resistant to natural biodegradation by microorganisms. This practice emits significant amounts of CO₂ and causes environmental pollution problem. This study investigates the potential of converting these wastes into renewable biohydrogen energy.

1.6 Aims

This work aims to model and optimize biohydrogen production from pretreated napier grass using immobilized mixed microbial consortium.

The specific objectives are:

1. Modeling and optimization of four hybrid pretreatment techniques for maximum release of fermentable sugars namely xylose and glucose from napier grass
2. Modeling and optimization of biohydrogen production from the pretreated napier grass using immobilized mixed microbial consortia
3. Semi-pilot scale biohydrogen production using napier grass substrate under optimized process conditions.
4. Analysis of the microbial community structure involved in hydrogen production in the semi-pilot scale reactor using 16rRNA gene sequencing analysis.

1.7 References

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CHAPTER 2

Napier grass (*Pennisetum purpureum*): a potential substrate for fermentative biohydrogen production – A review

2.1 Abstract

The increasing energy demand and consumption has led to fossil fuels depletion, global warming with alarming impacts on mankind. Biohydrogen is a potential clean energy carrier due to its high energy content and non-polluting nature. Lignocellulosic substrates are amenable for fermentative hydrogen production. Lignocellulosic napier grass holds potential as a valuable source of fermentable sugars because of its high holocellulose content, high biomass yield and the local availability. This review focuses on the potential of napier grass for fermentative hydrogen production. Furthermore, the influences of different pretreatment strategies on reducing sugar and hydrogen yields from pretreated feedstock are outlined. Finally, the effects of different process parameters on fermentative hydrogen production from napier grass and other lignocellulosic substrates are discussed.

Keywords: Napier grass, Biohydrogen, Pretreatment, Lignocellulose, Fermentation, Biomass

2.2 Introduction

The current reliance on fossil fuels derived energy has led to the depletion of fossil fuels reserves, environmental pollution and increased carbon emissions at global scale (Lo *et al.*, 2009a). Global CO₂ emissions are expected to increase from 31Gt in 2011 to reach approximately 37Gt by 2035 in response to increasing global energy demand (IPCC, 2013). Consequently global average temperature increase could exceed 5°C (Stern, 2008). This will have disastrous impacts on basic elements of human life affecting access to water supply, food production, human health and the environment. According to the World Health Organization (WHO), millions of people die yearly due to climate changes related causes (WHO, 2008). WHO predicts that between 2030 to 2050 climate changes will cause 250 000 additional deaths per year due to causes like malnutrition, malaria, diarrhea and heat stress (WHO, 2008). These concerns have strengthened the global search for renewable and environmentally friendly energy sources.

According to Dunn, (2002), hydrogen will play a major role in global renewable energy supply by 2100. Biohydrogen is emerging as a promising alternative fuel due to its social, economic and environmental benefits (Das and Veziroglu, 2001). Hydrogen can be produced through physicochemical and biochemical processes. Dark fermentative hydrogen production is a promising production route because it uses a wide range of substrates, simple reactor configurations, has high hydrogen production rates and low energy input (Holladay *et al.*, 2009; Valdez-Vazquez *et al.*, 2005; Wang and Wan, 2008). The commercialization of biohydrogen is hindered by its high production costs (Cheng *et al.*, 2011a). The use of lignocellulosic substrates for fermentative biohydrogen production could lower the production costs since these feedstocks are cheap, abundantly available and sustainable (Cheng *et al.*, 2011b). Biomass selection for fermentative hydrogen production depends on feedstock availability, fermentable sugars content and cost feasibility (Kapdan and Kargi, 2006). Globally approximately 150 to 170 x10⁹ tonnes of lignocellulosic biomass is produced per annum (Pauly and Keegstra, 2008). South Africa alone produces about 18 million tonnes (Mt) of lignocellulosic biomass per annum (Lynd *et al.*, 2003). Lignocellulosic substrates such as grasses have promising potential as feedstocks for fermentative biohydrogen production (Morandim-Grannetti *et al.*, 2013). Grasses have a relatively low lignin content which is about 27%, high holocellulose content up to 66.56% and are not key food sources for human consumption (Hamelinck *et al.*, 2005).

Napier grass (*Pennisetum purpureum*) is a valuable source of fermentable sugars for microbial conversion into biofuels and biomaterials (Wongwatanapaiboon *et al.*, 2012). It is rapid growing, invasive, C₄ perennial, native and abundant in African grasslands, high biomass yielding crop with high adaptability, pathogenic resistance abilities and high moisture content (DiTomaso *et al.*, 2010; Smith *et al.*, 2013). Globally, Somerville *et al.* (2010) reported annual cumulative dry matter napier grass yields of 85 tons per hectare and an annual cumulative yield of 40 tons per hectare has been recorded in South Africa (Reddy *et al.*, 2012). The major advantages of napier grass based fuel is the local availability of the biomass, renewability and the feasibility of biomass conversion without high capital costs (Hoogwijk *et al.*, 2003).

Napier grass is composed of cellulose, hemicellulose and lignin polymers tightly packed together through ester, ether and hydrogen bonds (Nissila *et al.*, 2014). These linkages limit biomass hydrolysis for fermentable sugars release. Thus, napier grass requires preliminary pretreatment for fermentable sugars solubilization prior to microbial hydrogen fermentation. Different pretreatment strategies for napier grass hydrolysis for fermentable sugars and biohydrogen yields have been assessed (Mosier *et al.*, 2005).

This paper reviews the potential application of napier grass as a feedstock for fermentative hydrogen production. The effects of different pretreatment methods on fermentable sugars and hydrogen yields from the pretreated feedstock are discussed. Finally, the influences of process parameters on dark fermentative hydrogen production from napier grass and other lignocellulosic substrates are elaborated.

2.3 Composition and production of napier grass

Napier grass is composed of interlinked polymers of about 35-45% cellulose, 25-40% hemicellulose and 20-35% lignin (Rekha and Aniruddha, 2013). Its composition varies from place to place and seasonally. Its composition is also largely influenced by harvest times, crop inputs, methods of analysis and analyzing extract-free napier grass (non-structural carbohydrates are removed) or non-extract free biomass (Saxena *et al.*, 2009). The chemical composition of napier grass is presented in table 1.

Table 2.1 - Chemical compositions of napier grass

Cellulose %	Hemicellulose %	Lignin %	Holocellulose %	Reference
34.12	36.34	30.40	-	Morandim-Grannetti <i>et al.</i> (2013)
-	-	26.2	66.59	Lee <i>et al.</i> (2010)
32.92	36.46	3.6	-	Wongwatanapaiboon <i>et al.</i> (2012)
32	20	9	-	Saxena <i>et al.</i> (2009)
45.66	33.67	20.60	-	Reddy <i>et al.</i> (2012)

-: no data

Napier grass dry matter yields of up to 40 tons/ha/year have been reported in South Africa. The dry matter yields depend on growing conditions namely soil type, temperature, fertilizer input etc. (Reddy *et al.*, 2012; Holtzapple *et al.*, 1994). Napier grass growth begins at temperatures above 15°C with optimum growth temperature between 25 to 40°C (Russell and Webb, 1976). This grass grows best in areas with high rainfalls above 1500mm per year (Russell and Webb, 1976). It has deep root system which enables drought tolerance. Comparative studies on napier grass yields with and without fertilizer supplements show that fertilizer addition improves dry matter yields (Vicente-Chandler *et al.*, 1959). Highest napier grass yields of 84 800kg DM/ha/year has been reported under natural rainfall of 2000mm per year in Kenya (Reddy *et al.*, 2012). Yields of 35 500, 32 400 and 20 800kgDM/ha/year have been reported in Tobago, Colombia and Nigeria (Walmsley *et al.*, 1978; Moore and Bushman, 1978; Adegbola, 1964).

2.4 Factors limiting napier grass hydrolysis

Napier grass does not only have the advantages of high biomass yield and local availability, but it also has high carbohydrate content which is favorable for cost friendly biohydrogen production (Wongwatanapaiboon *et al.*, 2012). However like other lignocellulosic biomass, it is resistant to direct biodegradation by microorganisms and enzymes. This is due to its composition and polymer interactions within the biomass which renders recalcitrant biomass structure thus necessitate a pretreatment step to break up these interactions for the production of monomeric sugars. Additionally, factors such as steric hindrance of lignin due to substitution patterns, polymer-enzyme interaction (Kumar *et al.*, 2008), degree of lignification, ferulate-induced cross-linking of hemicellulose, lignin and protein components also influence napier grass hydrolysis

for fermentable monomeric sugars production (Liong *et al.*, 2012; Mosier *et al.*, 2005).

The lignin is made up of a framework of phenyl propane units namely coniferyl, guaracyl, syringyl and syringyl alcohol. High concentrations of these phenyl propane units gives biomass rigidity, protection against pathogen attacks, provides strength, water proof and hindrance to hydrolysis by forming a steric barrier limiting enzymatic penetration (Kumar *et al.*, 2008). The lignin barrier also causes non-specific binding of cellulases thus reducing productive cellulose hydrolysis of biomass due to low polymer-enzyme interactions (Kumar *et al.*, 2012; Mood *et al.*, 2013). Ferulate-induced cross-linking of hemicellulose, lignin and protein components are thought to limit degradation of napier grass, however unambiguous evidence is lacking (Faulds *et al.*, 2002). When napier grass was pretreated with ferulate cross-links cleaving chemicals, hydrolysis was enhanced (Faulds *et al.*, 2002). Correlative studies are limited due to high variability of the ferulates incorporated into hemicellulose and lignin (Kumar *et al.*, 2008). Physico-chemical, physical, chemical and enzymatic strategies have been used for the conversion of napier grass to fermentable monomeric sugars (Liong *et al.*, 2012; Mosier *et al.*, 2005). Challenges in using napier grass as a fermentable feedstock include high costs of hydrolytic enzymes (cellulases), inhibitory compounds production, internal reactor structure corrosion, low sugar yields and high energy costs.

2.5 Pretreatment strategies to enhance microbial hydrolysis of napier grass

The pretreatment processes decompose polymeric components of lignocellulosic biomass thus forming simple monomeric sugars (Zilliox and Debeire, 1998). This enhances the accessibility of fermentable sugars to enzymatic hydrolysis (Kumar *et al.*, 2009). An effective pretreatment strategy must be economically feasible, reduce biomass particle size and have high yields of fermentable sugars. Additionally, it must not produce toxic byproducts mostly phenolic compounds that are inhibitory to downstream fermentation processes (Lynd *et al.*, 1996; Wyman, 1995, 1996, 1999; Delgenes *et al.*, 1996). Figure 2.1 illustrates the schematic diagram of fermentative hydrogen production from napier grass and other lignocellulosic substrates. A number of pretreatment strategies have been investigated for effective pretreatment of napier grass for hydrogen and reducing sugar yields. Table 2.1 shows the hydrogen yields and fermentable sugars production from napier grass and selected lignocellulosic feedstocks pretreated using different strategies.

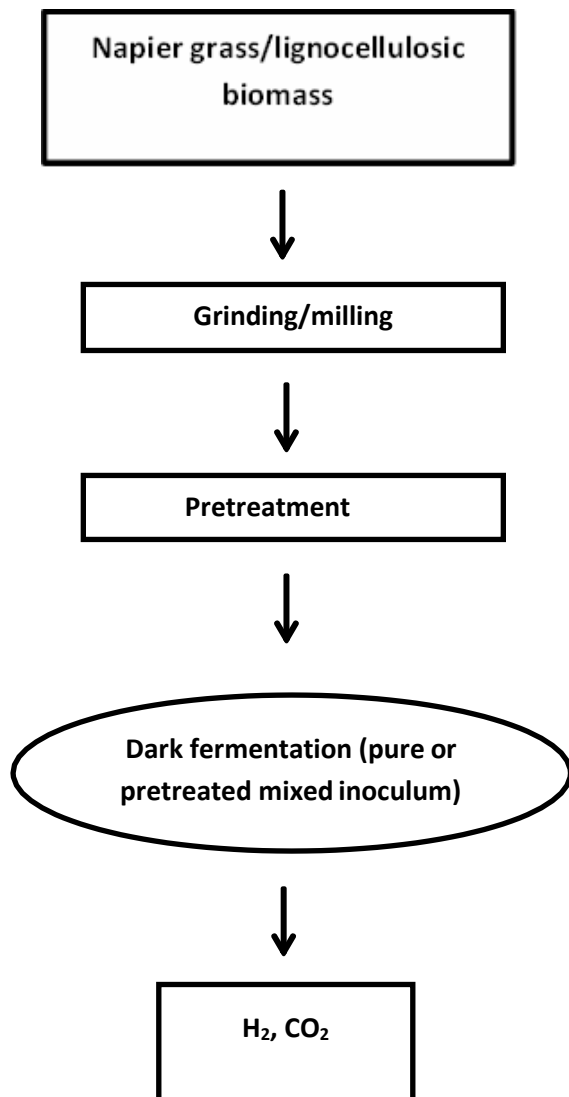


Figure 2.1 - A schematic diagram of fermentative hydrogen production from napier grass and other lignocellulosic biomass substrates.

2.5.1 Chemical pretreatment

Alkaline and acid pretreatment strategies are the most studied chemical pretreatment methods for hydrolysis of napier grass and other lignocellulosic substrates. Alkaline pretreatment has been used to effectively improve napier grass hydrolysis for biofuel production. Alkaline pretreatment uses bases namely NaOH, Ca(OH)₂, KOH and NH₃.H₂O to cleave lignin-carbohydrates linkages and solubilize lignin for fermentable sugars release from lignocellulosic biomass (Zheng *et al.*, 2014). NaOH is the most studied base in alkaline pretreatment, it has been used to pretreat corn stover, napier grass, wheat straw and sunflower stock (Zheng *et al.*, 2014).

Rekha and Aniruddha, (2013) studied the effect of NaOH concentration (0.3 to 0.9%), temperature (60 to 80°C) and time (60 to 180 min) on biogas production from napier grass. The authors observed significant increase of biogas and biomethane production (390ml/gTS and 171ml/gTS respectively) after napier grass pretreatment with 0.6%NaOH at 90°C for 120min compared to untreated napier grass where yields of 157ml/gTS and 46ml/gTS respectively were reported. This indicates that NaOH pretreatment improved biogas yields from the biomass. A comparative study of napier grass pretreated with NaOH and HCl has been reported by Cui and Shen (2012). The pretreated and untreated substrates were used in dark fermentative hydrogen production. A cumulative hydrogen volume of 19.25 ml was observed from the 0.5% NaOH pretreated feedstock compared to 4.38 ml from untreated napier grass, thus a 4.39 fold increase after pretreatment. For acid pretreatment a maximum cumulative volume of 72.21 ml was observed when napier grass was treated using 4% HCl, thus a 16.72 folds increase to untreated biomass. Further investigation showed that a 4% HCl had a 3.75 fold higher cumulative hydrogen production than 0.5% NaOH, thus suggesting HCl as a better pretreatment strategy for napier grass. Napier grass pretreatment with biological (using *Phanerochaete chrysosporium*) and alkaline pretreatment methods for bioethanol production have been reported by Liong *et al.*, 2012. The biologically and alkaline pretreated feedstocks were comparatively used for bioethanol production. The authors observed glucose yields of 0.74g glucose/g substrate and 0.43g glucose/g substrate for 7% NaOH and *Phanerochaete chrysosporium* pretreated napier grass respectively, thus a 0.31 higher glucose from alkaline pretreated napier grass. This suggests alkaline pretreatment as a better pretreatment method for napier grass hydrolysis to biological pretreatment.

Shamsuddin, (2013) optimized alkaline pretreatment conditions of napier grass for maximum

lignin solubilization. Napier grass was pretreated using 5 to 10% NaOH concentrations at temperatures 20 to 60°C for 30 to 90 minutes. A maximum lignin solubilization of 56.27% was achieved using 7.29% NaOH at 43°C for 75 minutes with 11.34mg/ml reducing sugar yield. This implies that NaOH pretreatment improved solubilization of lignin from the biomass. Although alkaline pretreatment of napier grass using NaOH is cost effective and efficient, it however causes Na⁺ ion inhibition during anaerobic digestion for biohydrogen production. Furthermore, disposal of Na⁺ containing effluent from fermentation systems could result to negative environmental impacts such as water pollution and soil salinization.

Acid hydrolysis of biomass uses organic and inorganic acids namely HCl, H₂SO₄, HNO₃, H₃PO₄ and maleic acid under varied concentrations ranging from 0.1 up to 70% to solubilize lignin for fermentable sugars generation from biomass. The efficiency of acid pretreatment majorly depends on the type of acid, concentration of acid, solid to liquid ratio, temperature and duration. High acid concentrations (from 30% up to 70%) have been reported as most effective in lignin solubilization however these concentrations are dangerous, toxic and result in bioreactor internal structures corrosion (Behera *et al.*, 2014). Therefore it is more economical to use dilute acid (concentrations less than 10%) hence extensive research studies have been conducted using dilute acid for biomass hydrolysis. Dilute acid pretreatment has been observed to generate lower concentrations of inhibitory compounds namely phenols than concentrated acid pretreatment (Behera *et al.*, 2014). It is also less toxic, hazardous and corrosive (Sun and Cheng, 2002).

H₂SO₄ is the most commonly used acid for biomass pretreatment. Techno-economic analysis of pretreatment strategies of lignocellulosic biomass suggested dilute acid pretreatment as most economical and practical for industrial scale application (Eggeman and Elander, 2005). Dilute acid pretreatment has also been observed to have consistent high release of fermentable sugars and short residence times. Orozco *et al.* (2007) studied the influence of dilute acid pretreatment on the hydrolysis of napier grass. Napier grass was pretreated with 2% H₂SO₄ at 90°C for 90 minutes. A reduction in hemicellulose and lignin concentrations from 20.9 to 13.5% and from 19.4% to 13.4% respectively was observed after dilute acid pretreatment. Acid pretreatment hydrolyzes mostly hemicellulose component of the biomass. Hemicellulose degradation releases simple products namely xylose, mannose, galactose and acetic acid. Industrial processes currently use pretreatment temperatures above 150°C, these high temperatures result in further sugar degradation to furfural, hydroxymethyl furfural, levulinic acid and formic acid (Aguilar *et*

al., 2002; Cao *et al.*, 2009). These compounds are inhibitory to downstream fermentative biohydrogen production. Therefore quantification and removal of these compounds is necessary, however this increases process costs (Aguilar *et al.*, 2002).

2.5.2 Physico-chemical pretreatment methods of napier grass

Steam explosion is the only physico-chemical pretreatment strategy that has been investigated for napier grass pretreatment. The efficiency of steam explosion is a function of pretreatment duration (how long the steam/biomass interaction is maintained), temperature, particle size and moisture content of lignocellulosic biomass (Duff and Murray, 1996). In steam explosion, biomass is mixed with either water or catalyzed with acid or base and exposed to high temperatures ranging from 160 to 260°C, high pressure ranging from 0.69 to 4.83 MPa for a short duration less than 30 min (Duff and Murray, 1996). The combination of high pressure, temperature and short duration causes a disruption of the fibrous structure of biomass thus improving biomass hydrolysis (Teymouri *et al.*, 2005).

The study of enzymatic hydrolysis of steam exploded napier grass using Artificial neural network (ANN) and regression analysis has been reported by Chang *et al.* (2011a). The input variables were three steam explosion parameters namely temperature ranging from 160°C to 210°C, reaction time from 2 to 20 minutes and particle size (1 and 5mm). The authors observed a reduction in glucan, xylan and lignin (67.3, 6.83 and 20.02% respectively) at 210°C for 20 minutes from 1mm particle size. A decrease in temperature and time to 160°C for 2 minutes resulted in a significant decrease in glucan, xylan and lignin components (47.3, 22.5 and 10.8%). The results showed that steam explosion temperature was the most significant parameter among the studied variables. Steam explosion however, results in xylan fraction destruction, inhibitory phenolic compounds production, incomplete lignin-carbohydrate matrix disruption and has high energy requirements (Mackie *et al.*, 1985). Steam explosion coupled with acids or bases namely H₂SO₄ and NaOH has been observed to generate inhibitory phenolic compounds derived from carbohydrate degradation. These phenolic compounds have been reported as detrimental compounds to biofuel producing microorganisms in anaerobic digestion phase. Thus suitable detoxification strategies are necessary after biomass pretreatment preliminary to anaerobic digestion.

2.5.3 Biological pretreatment of napier grass

Biological pretreatment uses microorganisms and their enzyme systems for the hydrolysis of lignin from biomass to expose carbohydrates for enzymatic hydrolysis for biofuel and biomaterial production. Compared to chemical and physico-chemical hydrolysis, enzymatic pretreatment is more cost effective since it is conducted under mild conditions (at temperatures less than 50°C and pH around 4.8) (Duff and Murray, 1996). Fungal and microbial species hydrolyze cellulose using cellulases enzyme systems. The cellulases are composed of endoglucanases, exoglucanases and cellobiases which work synergistically for cellulose hydrolysis (Sun and Cheng, 2002). The endoglucanases cleave the β -1-4 glycosidic bonds of cellulose thus creating free chain-ends in cellulose. The exoglucanases cleaves on the free chain-ends to release cellobiose which is hydrolyzed by the cellobiases into glucose (Sun and Chen, 2002). Xylan hydrolysis which is the major polymer in hemicellulose is catalyzed by xylanase enzyme systems. These xylanases are composed of endoxylanase, exoxylanase and xylosidase. The endoxylanase hydrolyses the 1-4 bonds between D-xylose of heteroxylans and xylo-oligosaccharides. The exoxylanases cleave the free chain-end of xylan thus releasing xylobiose which is hydrolysed by xylobiase into xylose. The different substituted groups in xylans are hydrolyzed by accessory enzymes namely the arabinofuranosidase, glucuronidase and acetylxyylan esterase (Saha, 2003). In many enzymatic biomass hydrolysis studies enzyme inhibition has been observed to negatively affect the efficiency of enzymatic hydrolysis processes thus optimum enzyme doses depending on the properties and concentration of each substrate must be investigated and used.

Biological hydrolysis of napier grass has been studied and reported by Lo *et al.* (2009b). The authors used thermophilic bacterial isolate (*Clostridium* strain TCW 1) for the hydrolysis of α cellulose, napier grass and bagasse for biohydrogen production. After hydrolytic experiments, the total reducing sugars concentrations of 1.22, 1.28 and 4.52 g/l were reported from napier grass, bagasse and α cellulose respectively. Maximum hydrogen yields of 7.40, 6.94 and 2.79mmol H₂/g reducing sugars were reported using pretreated hydrolyzates from napier grass, bagasse and α cellulose as substrates for biohydrogen production by *Clostridium butyricum* CGS5. Hence the biological pretreatment was observed efficient for reducing sugar yields and biohydrogen production from napier grass, bagasse and α cellulose.

Wen *et al.* (2014), investigated the effect of biological pretreatment of napier grass by three different microbial consortia through concurrent saccharification and anaerobic digestion. Comparative analysis of the efficiencies of the three microbial consortia was made based on degradation ability, sugars and biogas yields. The biomass pretreated using microbial consortia from plant litter and soil (dominant species: *Coprinus cinereus* and *Ochrobactrum sp.*) gave highest sugars and biogas yields 43.4% and 279ml/gVS respectively. This was 1.49 times higher than the untreated control. These results suggested that biological pretreatment is capable of significantly enhancing sugar and biogas yields from napier grass. Application of biological pretreatment of biomass is however limited by the long pretreatment duration thus making it unviable for commercial application. Research for industrial application of this pretreatment strategy is still needed to address key concerns such as cost feasibility, improving sugar yields, selectivity and efficiency (Zheng *et al.*, 2014).

2.5.4 Pretreatment combinations on napier grass hydrolysis

Napier grass hydrolysis by physico-chemical, chemical and biological strategies has been investigated. Studies revealed that each of the single pretreatment gives limited sugar yields due to specific mode of action (namely HCl only targets hemicellulose primarily, but not lignin) and intrinsic disadvantages. To overcome this, combined pretreatment methods which are the use of two or more pretreatment techniques on biomass are now being explored. The pretreatment combinations have been reported to be beneficial in enhancing sugar yields, reducing pretreatment duration and increasing severity. However, their use could increase pretreatment costs. Economic analysis is therefore key to assess biohydrogen production cost from napier grass pretreated using combined pretreatment methods. Lo *et al.* (2009b) combined biological and temperature-shift-enhanced pretreatment techniques for reducing sugar production from napier grass. Reducing sugar yields of 0.184g/l were observed from napier grass hydrolyzates pretreated using temperature shift strategy (35 to 45°C) and *Clostridium butyricum* CGS5 compared to using a constant temperature of 35°C where 0.036g/l reducing sugars was obtained. Thus it was concluded that pretreatment hybridization enhances reducing sugar production. Redding *et al.* (2011) studied the influence of high temperature and dilute acid combination on bermuda grass hydrolysis. Dilute H₂SO₄ concentration range 0.3 to 1.2%, high temperature range 120 to 180°C and pretreatment duration from 5 to 60 min was investigated for optimal reducing sugars generation from bermuda grass. The authors observed maximum reducing sugar yield of 94%

using 1.2% H₂SO₄ at 140°C for 30 minutes.

Yu *et al.* (2013) combined ozone and soaking aqueous ammonia for the pretreatment of lawn grass for enhanced reducing sugar production. The ozonation reaction was performed under 5.3% ozone (0.79g O₃ /g TS) at a flow rate of 2000ml/min for 10min. The hydrolyzed mixture was further soaked in 30% ammonia hydroxide solution at 50°C for 24 hours. Maximum sugar recovery of 89.63% reducing sugar was observed after the combined pretreatment compared to 48.50 and 56.71% reducing sugars observed after ozone and soaking aqueous ammonia pretreatment respectively were applied. Bohorquez *et al.* (2014) studied the effect of peroxide and enzymatic pretreatment combinations on the reducing sugar yields from napier grass. The authors observed reducing sugar yields of 287.8mg/g and 245.81mg/g glucose and xylose respectively. It was therefore concluded that peroxide and acid pretreatment combinations enhanced the reducing sugar yields from napier grass.

Table 2.2 - Reducing sugar production and hydrogen yields from napier grass and selected lignocellulosic substrates pretreated using different pretreatment strategies.

Type of substrate	Pretreatment strategy	Inoculum source	Reactor configuration	Reducing sugars (g/l)	Hydrogen yields (mol H ₂ /mol fermentable sugars)	Reference
Wheat straw	Hydrothermal,180	Mixed culture	Continuous system	-	1.43	Kongjan <i>et al.</i> 2010
Napier grass	Biological, <i>Clostridium</i> TCW1	<i>Clostridium butyricum</i>	Batch system	0.74	1.33	Lo <i>et al.</i> 2009a
Corn stover	Steam explosion, 180	Digested sludge	Batch system	-	2.84	Datar <i>et al.</i> 2007
Rice straw	55% H ₂ SO ₄ ,	Mixed culture	Batch system	24.5	0.44	Liu <i>et al.</i> 2013
Corn stover	dilute H ₂ SO ₄ ,150°C, 60 min	mixed culture	Batch system	21.7	1.53	Liu and Cheng, 2010
Rice straw	3% H ₂ SO ₄ , 121°C for 60 min	Mixed culture	Batch system	33.2	1.89	Chang <i>et al.</i> 2011a
Sugarcane bagasse	1% H ₂ SO ₄ , 45 min	Elephant dung	Batch system	11.3	0.84	Fangkum and Reungsang, 2011
Palm oil trunk	1.5% H ₂ SO ₄ , 450Watts for 7.5 min	Hot spring sediment	Batch system	21.8	0.71	Khamtib <i>et al.</i> 2011
Napier grass	7.5% H ₂ O ₂ and NaOH	<i>T. reesei</i> (xylanase and cellulase), <i>S. cerevisiae</i> and <i>P. stipitis</i>	Batch system	4.14	-	Wongwatanapaiboon <i>et al.</i> (2012)
Crystalline cellulose	Ionic liquid exchange	<i>Thermotoga neopolitana</i>	Batch system	-	1.22	Nguyen <i>et al.</i> 2008
Oat straw	Enzymatic (cellulase)	Anaerobic sludge	Batch system	-	0.81	Arreola-Vargas <i>et al.</i> 2013
Rice straw	55% H ₂ SO ₄ , 40°C, 120 min	Mixed culture	Continuous system	-	0.69	Liu <i>et al.</i> 2013
Lawn grass	4% HCl	Cracked cereal	Batch system	-	7.2L/kg dry substrate	Cui and Shen, 2012
Corn cob	1% HCl	Dairy manure mixed culture	Batch system	-	110L/kgTVS	Pan <i>et al.</i> 2010
Napier grass	7% NaOH, 4hours	-	Batch system	15.2g/g	-	Liong <i>et al.</i> 2012
Napier grass	<i>Phanerochaete chrysosporium</i> , 3 weeks	-	Batch system	18.4g/g	-	Liong <i>et al.</i> 2012
Napier grass	7% NaOH, 43°C, 75minutes	-	Batch system	11.34mg/ml	-	Shamsuddin, 2013
Napier grass	Temperature shift and <i>Clostridium</i> sp.	-	Batch system	0.184	-	Lo <i>et al.</i> 2009b
Napier grass	Temperature(35°C)	-	Batch system	0.036	-	Lo <i>et al.</i> 2009b

2.6 Biohydrogen production from napier and other lignocellulosic substrates

2.6.1 Influences of pretreatment methods on fermentable sugars and hydrogen yields

The effect of pretreatment strategies on fermentable sugars yields as well as hydrogen yields from pretreated napier grass and other lignocellulosic substrates have been reported (Chandra *et al.*, 2007; Cheng *et al.*, 2011). Direct biomass fermentation has low sugar and hydrogen yields compared to pretreated biomass. Theoretical hydrogen yields from hexose sugars as the sole carbon source are 4mol H₂/mol hexose (Nissila *et al.*, 2014). The highest documented hydrogen yield on hexose sugar is 3mol H₂/mol hexose from lignocellulosic corn stover pretreated using hybrid pretreatments namely steam explosion and sulfuric acid (Datar *et al.*, 2007). The highest hydrogen yield reported from napier grass is 1.33mol H₂/mol hexose when the biomass was pretreated using moist heat and *Clostridium* TCW1 (Lo *et al.*, 2009b). These yields are high and cost competitive when compared to hydrogen yields observed from pure hexose (glucose) with mixed cultures from digested sludge, cow manure and pure culture *Caldicellulosiruptoc saccharolyticum* which gave 2.88, 2.56 and 3.60 mol H₂/mol glucose respectively (Wang and Wen, 2008; Yokoyama *et al.*, 2007; de Vrije *et al.*, 2007). Competitively high hydrogen yields have also been reported from biomass pretreated using single hydrothermal pretreatment. This strategy however has high energy demand thus is costly (Jung *et al.*, 2011). Biological pretreatments also have promising hydrogen yields however the long pretreatment durations and optimization of growth conditions limit industrial feasibility of this strategy (Datar *et al.*, 2007).

In contrast, low hydrogen yields with significant batch to batch variations have been reported from biomass pretreated using single strategies such as ionic liquid, alkaline and concentrated acid (Kapdan and Kargi, 2006). For the alkaline and concentrated acid pretreated biomass these low yields are likely due to the formation of inhibitory compounds (Kapdan and Kargi, 2006). The ionic liquid strategy still requires further studying and optimization for higher hydrogen yields. Further studies on the optimization of pretreatment methods or combinations are necessary for optimum fermentable sugars and hydrogen yields from napier grass and other lignocellulosic substrates.

2.7 Influences of process parameters on hydrogen fermentation

The direct conversion of napier grass and other lignocellulosic feedstocks to hydrogen is hindered by substrate composition limiting readily hydrolysis of biomass by microbial enzymes. This is because the conditions for optimum lignocellulose hydrolysis are different from the conditions for optimum hydrogen production. For example, optimum pH for efficient lignocellulose hydrolysis has been reported near neutral while for hydrogen production the reported optimum is between pH 5.0 to 5.5 (Calli *et al.*, 2008; Li and Fang, 2007). The influences of different physico-chemical parameters affecting hydrogen production for napier grass and other lignocellulosic biomass are detailed.

2.7.1 Temperature

Hyperthermophilic (above 70°C), thermophilic (between 50 to 60°C) and mesophilic (between 20 to 40°C) microbial cultures have been studied for hydrogen production from fermentable sugars from napier grass and other lignocellulosic substrates. Cakir *et al.* (2010) studied the effect of temperature on hydrogen production from acid pretreated lignocellulosic wheat straw. The authors observed that increasing temperature from the mesophilic to the thermophilic ranges increases hydrogen yields and shortens the lag phase duration. Similar observations have been made from lignocellulosic bagasse pretreated using a combination of moist heat and enzymatic hydrolysis (Chairattanamakorn *et al.*, 2009). A study by Nissila *et al.* (2012), reported highest hydrogen yield from concentrated acid pretreated lignocellulosic pulp at 28°C. The authors observed that a change in temperature to any value above or below 28°C, affected the distribution of soluble metabolites thus resulted in low hydrogen yields.

2.7.2 pH

The optimal pH values for maximum hydrogen production from carbohydrates have been reported to range from 5.2 to 7.0 (Lin and Fang, 2007). Initial pH values of 5 and 9 have been observed to give low hydrogen yields. For each fermentation process the initial pH depends on the hydrogen producing community used. Most studies have investigated the effect of initial pH with no further pH control throughout the fermentation duration. For lignocellulosic biomass fermentation initial pH values of 6.0 and 7 with mixed culture from anaerobic sludge and cow compost respectively have been reported (Zhang *et al.*, 2007; Fan *et al.*, 2006). Optimum initial

pH values of 5.5 and 8.0 have also been reported with *Clostridium butyricum* and dairy manure mixed bacterial culture respectively (Pattrā *et al.*, 2008; Pan *et al.*, 2010). These initial pH values only indicate suitable start pH regions; these however do not indicate optimum pH for hydrogen production. Therefore, studies under controlled pH are necessary for optimum hydrogen yields.

2.7.3 Hydraulic retention time (HRT)

Hydraulic retention time (HRT) is an important parameter in hydrogen production from napier grass and other lignocellulosic biomass. It determines utilization of the substrate. For continuous hydrogen production from solubilized organic matter, HRT values ranging from 8 to 12 hours have been reported suitable (Shin *et al.*, 2004). Studies by Veeravalli *et al.* (2014) for optimizing hydrogen production from steam exploded switch grass showed that lowering the HRT from 16 to 8 hours reduces the methanogenic population and increases the hydrogen producing *Clostridium sp.* population in a continuous system of hydrogen production. In contrast, studies by Won and Lau, (2011) and Lui *et al.* (2008) observed no significant change in the methanogenic population when the HRT was decreased from 16 to 8 hours. The authors concluded that lowering the HRT must be coupled with lowering pH for effective reduction in the methanogenic bacterial population.

2.7.4 Inhibitory compounds

Subsequent to lignocellulosic biomass pretreatment through steam explosion, acid and alkaline pretreatment methods compounds such as furfural, hydroxymethyl furfural (HMF) and carboxylic acids have been observed to form. These are xylose and glucose oxidation products. Other phenolic compounds from partial lignin degradation have also been reported (Cao *et al.*, 2010). These compounds have negative effects on dark fermentative hydrogen production. The furfural and the HMF compounds lower microbial enzyme activity during dark fermentation. They also inhibit RNA and protein synthesis while degrading microbial DNA molecules (Liu *et al.*, 2004). The phenolic compounds irreversibly disrupt microbial cell membranes (Quemeneur *et al.*, 2012). While carboxylic acids tend to diffuse into microbial cells thus lowering intracellular pH which inhibits hydrogen production.

The influence of varying concentrations of furfural, HMF and other inhibitory compounds on hydrogen production from xylose with *Thermoanaerobacterium thermosaccharolyticu* W16 has been reported by Cao *et al.* (2010). The authors observed total hydrogen inhibition at furfural

and HMF concentrations above 1.5 and 2.0g/l respectively. Syringaldehyde inhibited hydrogen production at 1.0g/l while acetic acid and vanillin at 10 and 2g/l respectively. For heat shock pretreated anaerobic sludge, furfural concentration of 1,0g/l caused a significant decrease in hydrogen yield from xylose (Quemeneur *et al.*, 2012). The authors reported hydrogen yields of 0.51mol H₂/mol hexose with added furfural compared to 1.67mol H₂/mol hexose with no furfural addition. The addition of phenolic compounds had a minor effect on hydrogen yield as 1.28mol H₂/mol hexose was observed with added phenolic compounds compared to 1.67mol H₂/mol hexose with no phenolic compounds addition.

Detoxification is necessary to remove these inhibitory compounds before downstream dark fermentative hydrogen production. Total hydrogen inhibition was observed by Chang *et al.* (2011b) when concentrated acid hydrolyzed rice straw was used as a feedstock for hydrogen production. The authors then detoxified the pretreated rice straw with Ca(OH)₂ to remove furfural compounds and observed increased hydrogen yields. Detoxification uses chemical, physical and biological methods namely charcoal, cation exchange resin, activated carbon, overliming and yeast to remove inhibitory compounds after lignocellulosic biomass pretreatment (Sagnak *et al.*, 2011). Optimization of the detoxification conditions is essential for maximizing hydrogen yields from napier grass and other lignocellulosic biomass.

2.7.5 Biomass concentration

The hydrogen yield and the rate of hydrogen production increases with increasing biomass concentration however up to a certain concentration. Biomass concentrations above optimum set point result to the accumulation of volatile fatty acids (VFAs) which are known to inhibit hydrogen production (Sagnak *et al.*, 2011). The accumulation of VFAs lowers the pH in the fermentation medium to levels below optimum pH thus inhibiting microbial activity of hydrogen producers (Fan *et al.*, 2006). Furthermore, biomass concentrations above optimum tend to form higher concentrations of inhibitory compounds (Kongjan *et al.*, 2010). These also increase lag phase duration, cause substrate inhibition and also increase hydrogen partial pressure thus resulting to low hydrogen yields (Chu *et al.*, 2011). The influences of biomass concentration on hydrogen yield have been investigated in batch systems (Pan *et al.*, 2010). These studies observed at high substrate concentrations, the accumulation of VFAs, accelerated pH decrease, significant increase in the hydrogen partial pressure and very low hydrogen yields (Pan *et al.*, 2010). It is therefore necessary to optimize substrate concentration for maximum hydrogen yields.

2.8 Batch versus continuous fermentative hydrogen production from pretreated napier grass and other lignocellulosic substrates

Various studies on dark fermentative hydrogen production from napier grass and other lignocellulosic substrates have been reported under batch mode. The batch systems have the advantages of being easily operated and flexible over continuous system (Guo *et al.*, 2010). However, at an industrial scale context, continuous systems are recommended due to economic considerations and waste stock management. Continuous hydrogen fermentations from napier grass and other lignocellulosic substrates are scarcely reported. In the continuous and batch hydrogen production systems from lignocellulosic biomass, the highest reported hydrogen yield was 3.38mol H₂/mol hexose obtained from *Caldimonas taiwanensis* pretreated starch (Chen *et al.*, 2009) and 3.00mol H₂/mol hexose from steam exploded corn stover respectively (Datar, 2007). A study by Kongjan *et al.* (2010) observed low inhibitory compounds concentrations in a continuous hydrogen production system from concentrated acid hydrolyzed lignocellulosic wheat straw. Continuous hydrogen production systems give higher hydrogen yields than batch production systems. Liu *et al.* (2013), reported a 1.5 times higher hydrogen yield from concentrated acid pretreated rice straw in a continuous system than in batch system. Veeravalli *et al.* (2014), studied the optimization of hydrogen production from steam-exploded switch grass in an upflow anaerobic sludge blanket using a mixed anaerobic culture. The optimum pH and HRT set points were 5 and 10 hours respectively with a hydrogen yield of 99.86ml/g TVS. Microbial community analysis revealed a 50% reduction in the abundance of methanogenic population when the HRT was lowered from 16 to 8 hours and pH decreased from 7 to 5. Thus the higher hydrogen yields in continuous hydrogen production systems could also be attributed to the suppression through washing-out of hydrogen consuming methanogens while retaining both spore and non-spore forming hydrogen producing microorganisms at low pH and HRT values.

Further studies need to focus on the optimization of significant process variables for fermentative hydrogen production from napier grass and other lignocellulosic substrate in continuous flow reactor systems. These significant process variables include biomass concentration, process pH, process temperature and HRT.

2.9 Influence of microbial communities producing hydrogen from napier grass and other lignocellulosic substrates

Very few studies have reported the effect of microbial community composition on hydrogen and sugar yields from napier grass and other lignocellulosic substrates.

2.9.1 Hydrogen production using pure cultures

Only a small fraction of microorganisms have the ability to directly convert napier grass and other lignocellulosic biomass into hydrogen. A study by Wang *et al.* (2008) demonstrated that *Clostridium acetobutylicum* X9 can ferment microcrystalline cellulose into hydrogen with a reported hydrogen production of 6.4mmolH₂/h/g dry cell. Levin *et al.* (2006) reported that *Clostridium thermocellum* 27405 generated 1.0mol H₂/mol glucose from delignified wood fibers. Generally, the use of pure cultures for hydrogen production at industrial scale is not recommended. This is because pure cultures require microbial strain isolation, culturing, purification and characterization before use which can be time consuming and labor intensive. Another major drawback is that only a small fraction of microorganisms can be cultured on synthetic medium.

2.9.2 Hydrogen production using mixed cultures

Mixed microbial cultures have been reported to be able to convert napier and other lignocellulosic biomass into biohydrogen (Lay, 2001; Ginkel *et al.*, 2001). These have greater conversion rates and utilize broad carbon sources compared to pure cultures (Fang *et al.*, 2002). Mixed microbial cultures exist in the environmental sources such as compost or anaerobic sludge. These are excellent for cellulose hydrolysis. The highest hydrogen yield reported is 18mmol H₂/g substrate from microcrystalline cellulose feedstock using heat-shocked anaerobic sludge (Liu *et al.*, 2003). Wang *et al.* (2008) used co-cultures of *C. acetobutylicum* X9 and *Ethanoigenens harbinense* B49 for simultaneous hydrolysis and hydrogen production from microcrystalline cellulose through dark fermentation. The authors obtained a hydrogen yield of 16.2mg H₂/g cellulose. Further studies on the process dynamics revealed that *E. harbinense* rapidly consumes the reducing sugars produced from cellulose hydrolysis by *C. acetobutylicum*. This enhanced the hydrolysis of cellulose which led to improved hydrogen production rate and yield.

2.9.3 Hydrogen production using immobilized microbial cultures

Microbial cell immobilization has been observed to significantly enhance biomass retention and hydrogen yields thus allowing optimum hydrogen production (Wu *et al.*, 2005). In continuous hydrogen production systems cell immobilization allows better hydrogen retention and stable operation even at high dilution rates. Studies on the use microbial cell immobilization on fermentative hydrogen production from napier grass and other lignocellulosic substrate are rarely reported. Nomura *et al.* (2013) studied isolation and characterization of novel hydrogen producing *Clostridium sp.* This hydrogen producing strain was immobilized onto copolymer PEG-b-PPG, a maximum hydrogen yield of 2.91mol H₂/mol glucose was observed. Thus a 6-fold higher yield to the suspended isolate (0.45 molH₂/mol glucose).

2.10 Other biofuels and biomaterials from napier grass

Lin *et al.* (2010) studied response surface optimization for ethanol production from napier grass using *Klebsiella oxytoca* THLCO409. The effect of fermentation duration, initial pH, yeast concentration and temperature on ethanol production from napier grass was evaluated. Maximum ethanol concentration of 472ppm was observed after 11 days of fermentation, at pH 7, 7.0g/l yeast extracts concentration with a temperature set point at 31°C. The optimal conditions and ethanol concentration observed in this study are indicative for the potential application of napier grass as a feedstock for ethanol production. Wongwatanapaiboon *et al.* (2012) evaluated the use of 8 different grasses as feedstock for bioethanol production by *Saccharomyces cerevisiae* and *Pseudomonas stipites*. The feedstocks were pretreated with 7.5% alkaline peroxidase, pH 11.5 for 24 hours at 35°C. Highest ethanol yield of 1.14g/l was observed from vetiver grass while 0.97g/l was observed from napier grass. Considering dry matter yields, napier grass was deemed a better feedstock since it has higher annual dry matter yields 2720.55L/ha/year compared to 1091.84L/ha/year for vetiver grass. Rekha and Aniruddha, (2013) reported a methane yield of 0.158m³CH₄/kgTS from napier grass pretreated using 0.6%NaOH, thus a 3-fold higher yield than for untreated napier grass (0.047m³CH₄/kgTS). These findings suggest potential application of napier grass for biomethane production.

2.11 Conclusion

Dark fermentative hydrogen production from napier grass is an appealing method for renewable hydrogen production. The direct use of napier grass and other lignocellulosic substrates is limited by the recalcitrant nature of lignocellulosic biomass. Thus suitable pretreatment strategies are necessary to hydrolyze biomass for fermentable sugars release. High fermentable sugars and hydrogen yields have been reported for napier grass pretreated using biological pretreatment method. This method however requires long pretreatment durations due to slow hydrolysis rate of cellulolytic enzymes, thus it is not a viable method for commercial scale application. Pretreatment combinations have the potential to enhance the yields of fermentable sugars, high hydrolysis rates and hydrogen yields from napier grass and other lignocellulosic substrates. Different combinations that are efficient and cost effective need to be investigated and optimized for optimum sugar production and hydrogen yields from napier grass and other lignocellulosic substrates. Different studies on napier grass and other lignocellulosic substrates for fermentative hydrogen production showed that pH, temperature, pretreatment strategy, biomass concentration and HRT are the most significant parameters on hydrogen yields. Studies have shown that mixed microbial consortia immobilization significantly enhances hydrogen yields. Future investigations should focus on the effects of pretreatment combinations on reducing sugars and hydrogen yields from napier grass and other lignocellulosic substrates. The fermentable sugars from napier grass and other lignocellulosic substrates can be channeled for the production of other biofuels and biomaterials. The production of biohydrogen energy from napier grass will have a significant contribution to the mitigation of environmental pollution.

2.12 References

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CHAPTER 3

Modeling and Optimization of Xylose and Glucose Production from Napier grass (*Pennisetum purpureum*) using Hybrid Pretreatment Techniques.

3.1 Abstract

Lignocellulosic biomass is an excellent potential substrate for renewable biofuel production. However its conversion into fermentable sugars is hindered by the interlinked polymers of cellulose, hemicellulose and lignin. Thus an efficient and cost effective pretreatment is required. This work models and optimizes four hybrid techniques of napier grass pretreatment for xylose and glucose production namely HCl and moist heat (HH), HCl and microwave (HM), NaOH and moist heat (NH) and NaOH and microwave (NM) using Response Surface Methodology (RSM). The coefficients of determination (R^2) of 0.83 and 0.97 were obtained for xylose and glucose production respectively using HH hybrid pretreatment, and 0.90 and 0.80 were obtained for xylose and glucose respectively using HM hybrid pretreatment. The optimized pretreatment conditions of HH gave 12.83g/l xylose and 2.28g/l glucose and HM optimized pretreatment gave 15.06g/l and 2.44g/l xylose and glucose. A xylose to glucose ratio of 5.6:1 was obtained for the optimized HH pretreatment compared to 6.1:1 for the optimized HM pretreatment. For NH and NM hybrid pretreatments, low concentration of fermentable sugars was observed (<0.5g/l). The findings indicate that xylose and glucose production from napier grass can be enhanced by an optimal combination of pretreatments of HCl and moist heat at 4.39% HCl, 93.07°C for 180 min, or using a combination of microwave and HCl at 5% HCl, 500 W for 30 min. The optimum generation of xylose and glucose from napier grass leverages its potential as substrate for the production of renewable biofuels and biomaterials.

Keywords: Napier grass, Lignocellulosic biomass, Lignocellulosic biomass pretreatment, process modeling and optimization, Biohydrogen production.

3.2 Introduction

Global climate change, escalating fuel prices, high global energy demand and depletion of fossil fuel reserves are driving the search for alternative energy sources. High dependency on non-renewable fossil fuel has resulted in a negative environmental impact through greenhouse gas emission, excessive climate change (Azwar *et al.*, 2014) and the depletion of the energy reserves at high pace (Fiddaman, 2002). Lignocellulosic biomass is gaining importance as a cleaner, readily available and renewable energy source (Fiddaman, 2002; Iroba *et al.*, 2013). About 200 billion tonnes of biomass are produced worldwide per annum (Zhang, 2008). Lignocellulosic biomass is a source of mixed fermentable sugars that can be used for biofuel and biomaterial production (Iroba *et al.*, 2013). It is composed of interlinked polymers of about 35-45% cellulose, 25-40% hemicellulose and 20-35% lignin (Rekha and Aniruddha, 2013). These interlinks of chemical bonds give biomass structural support, impermeability and resistance to oxidative stress and microbial attack (Kumar *et al.*, 2009). However, this limits the use of biomass as feedstock in biofuel and biomaterial production (Iranmahboon *et al.*, 2005). Hence efficient pretreatment procedures are required to disrupt these interlinks and expose cellulose and hemicellulose to hydrolytic enzymes for fermentable sugar release.

Steam explosion, alkali and acid hydrolysis, wet oxidation, ammonia fiber explosion, biological and microwave hydrolysis are some examples of biomass pretreatment strategies (Prakasham *et al.*, 2009). Steam explosion uses high temperature ranging from 160 to 260°C and a pressure ranging from 0.69 to 4.83MPa is rapidly applied to lignocellulosic biomass for a short duration (to promote hemicellulose hydrolysis) after which, the system undergoes explosive decomposition (Ramos *et al.*, 1992). The hydrolysis of napier grass through steam explosion has been reported by Chang *et al.* (2011) under varied temperature from 160 to 210°C and a reaction time between 2 to 20 mins. The author reported a significant reduction in glucan, xylan and lignin (67.3, 6.83 and 20.02% respectively) at 210°C for 20 mins. Steam explosion however, results in xylan fraction destruction, toxic and inhibitory phenolic compounds production, incomplete lignin-carbohydrate matrix disruption and has high-energy requirements (Mackie *et al.*, 2005). Biological pretreatment has been applied to hydrolyze lignocellulosic biomass (Zhang, 2008). This method uses fungal enzymes to degrade lignin, hemicellulose and polyphenols. Industrial application of this method is limited by slow degradation rate and

microorganisms consuming some hydrolyzed carbohydrate fraction. Acid pretreatment solubilizes lignin and hydrolyzes hemicellulose to xylose, thus making cellulose accessible to enzymatic hydrolysis (Eggeman and Elander, 2005). This strategy is inexpensive and efficient, however it causes corrosion to the bioreactor internal structures (Lopez-Arenas *et al.*, 2010). Alkaline pretreatment strategy disrupts the lignin structure of biomass but also removes uronic acid substitutions on hemicellulose thus reducing accessibility of hemicellulose to hydrolytic enzymes (Cardona and Sachnez, 2006). Microwave radiation and thermal pretreatment strategies disrupt lignin, reduce degree of polymerization of biomass and hydrolyze hemicellulose to xylose. These pretreatment strategies have high energy requirements and are slow (require long process times) (Lopez-Arenas *et al.*, 2010). Ammonia fiber explosion uses hot liquid ammonia (<90°C) under high pressure for specific duration (<30 min) to delignify and solubilize hemicellulose (Cardona and Sachnez, 2006). This process is highly efficient, however the costs of ammonia and recovery process make this pretreatment economically unviable (Cardona and Sachnez, 2006). Thus, more efficient and cost effective alternative biomass pretreatment approaches are investigated.

Hybrid pretreatment techniques of steam explosion and dilute 1% H₂SO₄ as preimpregnation agent on raw wheat straw have been reported, with yield improvement of 8 to 10g glucose/100g raw wheat straw compared to 6.4g glucose/100g wheat straw obtained without acid addition (Lopez-Arenas *et al.*, 2010). Ideally, biomass pretreatment strategy should be cost effective, economically viable, free from inhibitory by-products and be practical at large scale (Aden and Foust, 2009). Suhardi *et al.* (2013) combined biologic and dilute acid pretreatment strategies on energy cane hydrolysis for ethanol production, 3055mg/L ethanol yield was observed compared to 1725 and 1266mg/L ethanol observed for fungal and 2% H₂SO₄ treated energy cane.

The Response Surface Methodology (RSM) is a modeling and optimization technique that evaluates the interactive and synergistic effects of all input variables on the process to achieve a maximum output (Muthuvelayudham and Viruthagiri, 2010). RSM has been successful used in optimization of various bioprocesses (Hu *et al.*, 2004; Yue *et al.*, 2007), but its application for the determination of optimal set points for biomass pretreatment using hybrid techniques is scantily reported.

Napier grass (*Pennisetum purpureum*) has high cellulose content (36.46%) (Saxena *et al.*, 2009), rapid growth, invasive nature, high biomass yield (up to 40 metric ton/ha/year) and high adaptability (Chang *et al.*, 2011; Smith *et al.*, 2013; DiTomaso *et al.*, 2010). It is a C₄ perennial grass species widely distributed and native to African grasslands with high light, water and nitrogen utilizing efficiency (Somerville *et al.*, 2012). Napier grass has been recognized as an opportunistic weed outcompeting native vegetation in Central America. About \$22.5 million was spent by Florida government in 2005 for napier grass growth control (FDEP, 2005).

This work models and optimizes four hybrid pretreatment techniques for xylose and glucose production from napier grass namely HCl and heat (HH), HCl and microwave (HM), NaOH and heat (NH) and NaOH and microwave (NM). It further studies the interactive effects of pretreatment duration, pretreatment temperature and chemical concentration on xylose and glucose yields. Additionally, a preliminary assessment of fermentative hydrogen production using the optimally pretreated napier grass is carried out.

3.3 Materials and methods

3.3.1 Lignocellulosic biomass

The napier grass used as a substrate in this study was harvested at 6 months old from Grassland Science tunnels in the University of KwaZulu-Natal Pietermaritzburg campus, South Africa. It was dried at 60°C for 72 hours and reduced to particle size of 1mm using a centrifugal mill (Retsch ZM-1, Durban South Africa).

3.3.2 Experimental designs

Based on reported literatures on efficient biomass pretreatment techniques, HCl (0.1 to 5%), NaOH (0.1 to 5%), moist heat (60 to 100°C) and microwave intensity (500-1000 W) pretreatments were selected (Tables 3.1 and 3.2). Four hybrid techniques were considered, namely HCl and heat (HH), HCl and microwave (HM), NaOH and heat (NH), and NaOH and microwave (NM). Box-behken design was used to generate seventeen experimental runs with varied input parameters for each of the hybridized procedure, thus a total 68 experimental runs were carried out in duplicate.

Table 3.1 – Experimental conditions for HH and NH hybrid techniques

Independent variables	Symbols	Coded		
		-1	0	1
Acid/base concentration (%)	A	0.1	2.55	5.0
Pretreatment duration (min)	B	15	97.50	180
Pretreatment temperature (°C)	C	60	80	100

Table 3.2 – Experimental conditions for HM and NM hybrid techniques

Independent variables	Symbols	Coded		
		-1	0	1
Acid/base concentration (%)	A	0.1	2.55	5.0
Microwave intensity (W)	B	500	750	1000
Heating duration (min)	C	5	17.50	30

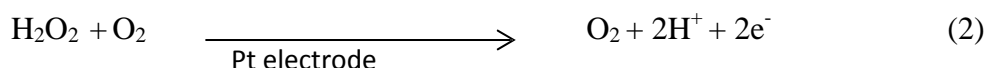
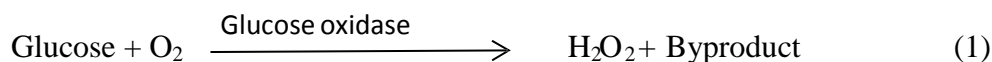
3.3.3 Experimental set up

Moist heat based pretreatments were conducted in PolysScience Analog water bath with variable temperature, ranging from 5 to 100°C. 10g of napier grass powder were immersed in 100ml dilute HCl or NaOH at concentrations (0.1%, 2.55% and 5 % (w/v)) with 10% solid loading. The mixtures were placed in sealed 500ml Scotch bottles and exposed to moist heat at temperatures and durations specified in the design.

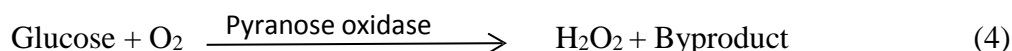
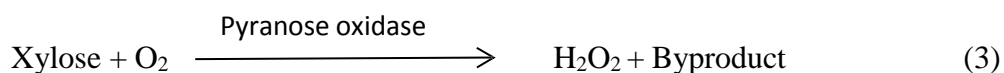
Microwave based pretreatments were carried out in a Defy microwave oven (model DMO353) which provided radiations at variable power levels (100 to 1500W). 10g of napier grass powder were immersed in 100ml dilute HCl or NaOH at concentrations stated in the design. The mixtures were placed in sealed 500ml Scotch bottles and exposed to microwave radiations (500, 750 and 1000W). The pretreatment duration was carried out as specified in the design (Tables 3.1 and 3.2).

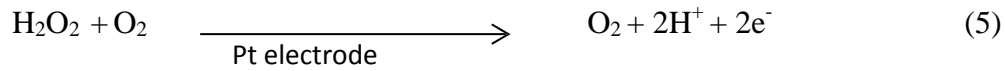
3.3.4 Analytical methods

The xylose and glucose produced after each hydrolytic experiment were determined simultaneously using a glucose analyzer (Model 2700 select-dual configuration, YSI USA). This system provides near real time data during saccharification. It is equipped with dual biosensors composed of three layers (polycarbonate layer, immobilized enzyme layer and cellulose acetate layer) attached to platinum electrodes. The immobilized enzyme layer in the first electrode is glucose oxidase which measures glucose concentration in range 0 to 9g/l using the following reactions:



The glucose concentration was directly proportional to the electron flow. The immobilized enzyme layer in the second electrode is pyranose oxidase which measures both xylose and glucose in detection range 0.5 to 30g/l using the following reactions:





Xylose and glucose concentrations were directly proportional to the electrons flow. Prior to measurements the machine was calibrated with 20g/l and 2.5g/l of xylose and glucose calibrator solutions respectively. The system calculated the xylose and glucose sensitivities and used the compensation equations to subtract interfering analytes.

The experimental data obtained were used to fit four different polynomial model equations relating xylose and glucose to the process input treatment conditions in hybrids pretreatment of HH and HM. The general form of the model is shown in Equation 6.

$$Y = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_3 X_3 + \alpha_{11} X_1^2 + \alpha_{22} X_2^2 + \alpha_{33} X_3^2 + \alpha_{12} X_1 X_2 + \alpha_{13} X_1 X_3 + \alpha_{23} X_2 X_3 \quad (6)$$

Where Y represents response output (glucose or xylose), 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 quadratic coefficients and $\alpha_{12} X_1 X_2$ to $\alpha_{23} X_2 X_3$ represents the interaction of coefficients. The significance of the model was assessed by Analysis Of Variance (ANOVA) using Design Expert software, (Stat Ease, Inc.). The optimum experimental set points for maximum xylose and glucose production in the hybrid pretreatment of HH and HM were obtained by solving the model equations and were subsequently validated.

3.3.5 Preliminary assessment of pretreated napier on biohydrogen production

3.3.5.1 Seed sludge

Anaerobic sludge obtained from the Darville wastewater treatment plant in Pietermaritzburg South Africa was used as the inoculum in the study. The sludge was stored at 4°C in the laboratory before use.

3.3.5.2 Batch fermentation experiments

Anaerobic sludge was boiled at 100°C for 30 min in a hot plate to inactivate the hydrogen consuming microorganisms and preserve the spore forming hydrogen producing bacteria. Fermentation experiments were carried out in duplicate in 250ml Erlenmeyer flasks. The flasks were inoculated with 25ml of the treated sludge and fed with 100ml of the optimally pretreated napier grass substrate and 125ml mineral salt solution to a total working volume of 250ml. The mineral salt contained (g/l): NH_4Cl 0.5, KH_2PO_4 0.5, K_2HPO_4 0.5, NaHCO_3 4.0, $\text{FeCl}_2 \cdot 2\text{H}_2\text{O}$

0.15, MgCl₂.6H₂O 0.085, ZnSO₄.7H₂O 0.01, MnCl₂.4H₂O 0.03, H₃BO₃ 0.03, CaCl₂.6H₂O 0.01, Na₂MoO₄.2H₂O 0.03. The pH of the fermentation medium was adjusted to 6.5 with NaOH pellets. Anaerobiosis was achieved by flushing the broth with nitrogen gas for 2 min and the flasks were tightly capped (Ramprakash and Muthukumar, 2015; Xu and Dushusses, 2015). They were incubated at 37°C in a shaking water bath at 100rpm for 72 hours.

The hydrogen fraction of the evolved gas was obtained using the hydrogen sensor (BCH-H₂, Bluesens, Germany). The sensor uses the thermal conductivity principle and measures hydrogen gas range from 0-100%. Water displacement method was used to measure the gas volume.

3.4 Results and discussion

3.4.1 Composition of napier grass

The composition of the napier grass used in the experiment is presented in Table 3.3. The high content of cellulose (28.88%) and hemicellulose (30.61%) evidences its potential as valuable source of fermentable sugars as earlier reported by Wongwatanapaiboon *et al.* (2012). The optimized hybrid pre-treatment of HM gave polymers solubilization of 18.96%, 83.3% and 8.35% (dry mass fractions) of cellulose, hemicellulose and lignin respectively. In the same pattern, the optimized hybrid pre-treatment of HH gave 15.9%, 76.8% and 21.2% solubilization of the dry mass fractions of cellulose, hemicellulose and lignin respectively (Table 3.3). Previous studies by Chang *et al.* (2011) reported a solubilization of 15.4% of hemicellulose with napier grass pretreated by steam explosion, thus a relatively low solubilization compared to the values achieved in the present study using the optimized hybrid pre-treatment strategies. HM hybrid pre-treatment showed higher solubilization of cellulose and hemicellulose compared to HH hybrid pretreatment with differences of 3% and 6.5% (dry mass fraction) respectively. However, the later gave a higher delignification compared to HM pretreatment with a difference of 12.9%.

Table 3.3. Napier grass composition before and after pretreatment

Sample	Cellulose (%)	Solubilization (%)	Hemicellulose (%)	Solubilization (%)	Lignin (%)	Solubilization (%)
Raw napier grass	28.88	-	30.61	-	9.34	-
HH pretreated napier grass	24.28	15.92	7.1	76.8	7.36	21.2
HM pretreated napier grass	18.96	34.35	5.1	83.34	8.56	8.35

-: no data

3.4.2 Modeling of HCl and moist heat hybrid pretreatment (HH).

Experimental results from the HH hybrid design (Table 3.4) were used to generate two polynomial models based on the general form of Equation 6. These models relate the input variables to glucose and xylose yields. The suitability of the models was assessed using the Analysis Of variance (ANOVA) (Table 3.5). The coefficients of determination (R^2) of 0.83 and 0.97 were obtained for xylose and glucose production respectively, thus 83% and 97% variations in the xylose and glucose production data respectively can be explained by the models. The low P-values (0.0443 and 0.0002) and high F-values (6.26 and 25.40) further confirm their significance.

Table 3.4 Xylose and glucose production from napier grass using HCl and moist heat hybrid pretreatment

Run	HCl (%)	Pretreatment duration (mins)	Pretreatment temperature (°C)	Xylose (g/l)	Glucose (g/l)
1	2.55	15.00	60.00	0.057	0.6055
2	2.55	97.50	80.00	10.5015	1.408
3	2.55	97.50	80.00	11.2435	1.4485
4	0.10	97.50	60.00	0.0165	0.245
5	2.55	97.50	80.00	8.7315	1.240
6	2.55	97.50	80.00	10.0945	1.162
7	5.00	97.50	80.00	14.1335	3.8655
8	5.00	97.50	100.0	6.606	4.7365
9	0.10	15.00	80.00	0.046	0.339
10	2.55	180.0	60.00	0.781	0.848
11	0.10	97.50	100.0	0.062	0.485
12	2.55	97.50	80.00	9.3685	1.1215
13	5.00	97.50	60.00	2.169	0.6615
14	5.00	15.00	80.00	0.561	0.4585
15	0.10	180.0	80.00	0.006	0.4055
16	2.55	180.0	100.0	9.3735	5.991
17	2.55	15.00	100.0	12.734	2.792

Table 3.5: Analysis of variance for xylose and glucose production generated using HH hybrid pretreatment.

Source	Sum of squares	df	Mean squares	F-value	P-value	R ²
Xylose Model	377.91	9	41.99	6.26	0.0443	0.8323
Glucose Model	44.73	9	4.97	25.40	0.0002	0.9703

df: degrees of freedom, F-value: Fisher-Snedecor distribution value, P-value: probability value, R²: coefficient of determination

$$\text{Xylose (g/l)} = 9.99 + 2.92 A + 1.36 B + 3.22C + 3.40 A B + 1.10 A C - 1.02 B C - 4.91 A^2 - 1.39 B^2 - 2.8 C^2 \quad (7).$$

$$\text{Glucose (g/l)} = 1.28 + 1.03 A + 0.86B + 1.46 C + 0.84 A B + 0.96 A C + 0.74 B C - 0.52 A^2 + 0.51 B^2 + 0.77 C^2 \quad (8).$$

The coefficients of estimates are shown in tables 3.6 and 3.7. Where A, B and C are linear coefficients of HCl concentration (%), pretreatment duration (min) and pretreatment temperature (°C) respectively, AB, BC and AC are interactive coefficients and A², B² and C² are square coefficients.

Table 3.6: Coefficient of estimate of the hybrid models and their confidence intervals for xylose production

Factor	Coefficient Estimate	df	Standard Error	95% CI	95% CI
				Low	High
Intercept	9.99	1	1.47	6.5	13.48
A	2.92	1	1.17	0.16	5.67
B	1.36	1	1.17	-1.4	4.12
C	3.22	1	1.17	0.46	5.98
AB	3.4	1	1.65	-0.5	7.3
AC	1.1	1	1.65	-2.8	5
BC	-1.02	1	1.65	-4.92	2.88
A²	-4.91	1	1.61	-8.71	-1.11
B²	-1.39	1	1.61	-5.19	2.41
C²	-2.86	1	1.61	-6.66	0.94

Table 3.7: Coefficient of estimate of the hybrid models and their confidence intervals for glucose production

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	1.28	1	0.2	0.81	1.74
A	1.03	1	0.16	0.66	1.4
B	0.86	1	0.16	0.49	1.23
C	1.46	1	0.16	1.09	1.83
AB	0.84	1	0.22	0.31	1.36
AC	0.96	1	0.22	0.44	1.48
BC	0.74	1	0.22	0.22	1.26
A²	-0.52	1	0.22	-1.03	-8.29 ⁻⁰³
B²	0.51	1	0.22	-5.84 ⁻⁰⁴	1.02
C²	0.77	1	0.22	0.26	1.28

df: degrees of freedom, 95% CI low: 95% confidence interval (low limit), 95% CI high: 95% confidence intervals (high limit)

3.4.3 Modeling of HCl and microwave hybrid pretreatment (HM)

Experimental result of HCl and microwave pretreatment (HM) (Table 3.8) were used to generate two polynomial process models based on the general form of Equation 6. These models relate the considered process variables to xylose and glucose yields respectively.

Table 3.8. Xylose and glucose production from napier grass using HCl and microwave hybrid pretreatment

Run	HCl (%)	Pretreatment duration (min)	Microwave intensity (W)	Xylose (g/l)	Glucose (g/l)
1	5	17.5	1000	14.5545	3.4705
2	0.1	5	750	0.053	0.229
3	2.55	17.5	750	2.6695	0.4995
4	5	30	750	14.6745	3.3885
5	2.55	30	1000	0.0475	0.0775
6	0.1	17.5	500	0.041	0.122
7	2.55	5	1000	15.292	2.683
8	5	17.5	500	10.9155	2.1535
9	0.1	30	750	0.1065	0.243
10	2.55	17.5	750	4.358	0.603
11	2.55	30	500	13.8805	3.0205
12	5	5	750	1.4455	0.3615
13	0.1	17.5	1000	0.076	0.1315
14	2.55	17.5	750	2.759	0.5355
15	2.55	17.5	750	3.484	0.5045
16	2.55	5	500	1.6195	0.3665
17	2.55	17.5	750	4.304	0.698

Experimental results from HM hybrid design (Table 3.8) were fitted into polynomial equations (equations 9 and 10) to generate process models

$$\text{Xylose (g/l)} = 3.19 + 4.70 A + 1.49 B + 0.11 C + 2.62AB + 2.01 AC - 6.41 BC - 0.80 A^2 + 1.70B^2 + 3.09C^2 \quad (9).$$

$$\text{Glucose (g/l)} = 0.73 + 1.14 A + 0.66 B + 0.11 C + 0.79 AB + 0.98AC - 1.16 BC + 0.27 A^2 + 0.26 B^2 + 0.51 C^2 \quad (10).$$

Where A, B and C represents linear coefficients of HCl concentration, pretreatment duration and microwave intensity respectively, AB, BC and AC are the interactive coefficients of parameters and A², B² and C² represents the quadratic coefficients. The optimum conditions for glucose and xylose production were obtained by solving the quadratic equations.

Analysis of variance (ANOVA) was performed to determine the suitability of the models (Table 3.9). The coefficients of determination 0.90 and 0.80 for xylose and glucose production were obtained respectively. This means that 90% and 80% variations observed in data can be explained by the models. The significance of the model was confirmed by the high F-values (6.95 and 5.12) and low p-values (0.0091 and 0.00737) for xylose and glucose production respectively. The coefficient of estimates are shown (tables 3.10 and 3.11), where A, B and C are the linear coefficients HCl concentration, pretreatment duration and microwave intensity respectively, AB, AC and BC are interactive coefficients and A², B² and C² are the square terms of experimental variables.

Table 3.9. Analysis of variance generated for xylose and glucose production from HM models.

Source	Sum of Squares	df	Mean Square	F-value	p-value	R ²
Xylose Model	458.55	9	50.95	6.95	0.0091	0.90
Glucose Model	27.64	9	3.07	5.12	0.00737	0.80

df: degrees of freedom, F-value: Fisher-Snedecor distribution value, P-value: probability value, R²: coefficient of determination.

Table 3.10. Coefficient estimates of the hybrid model and confidence intervals on xylose production.

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	3.19	1	1.21	0.33	6.05
A	4.7	1	0.96	2.44	6.96
B	1.49	1	0.96	-0.78	3.75
C	0.11	1	0.96	-2.15	2.37
AB	2.62	1	1.35	-0.58	5.82
AC	2.01	1	1.35	-1.19	5.21
BC	-6.41	1	1.35	-9.61	-3.21
A²	-0.8	1	1.32	-3.92	2.32
B²	1.7	1	1.32	-1.42	4.82
C²	3.09	1	1.32	-0.034	6.21

Table 3.11. Coefficient estimates of the hybrid model and confidence intervals on glucose production

Factor	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	0.73	1	0.44	-0.32	1.78
A	1.14	1	0.35	0.31	1.97
B	0.66	1	0.35	-0.17	1.49
C	0.11	1	0.35	-0.72	0.94
AB	0.79	1	0.5	-0.38	1.96
AC	0.98	1	0.5	-0.19	2.16
BC	-1.16	1	0.5	-2.33	0.012
A²	0.27	1	0.48	-0.87	1.41
B²	0.26	1	0.48	-0.88	1.41
C²	0.51	1	0.48	-0.63	1.65

df: degrees of freedom, 95% CI Low: 95% confidence intervals (low limit), 95% CI High: 95% confidence interval (high limit).

3.4.4 Effect of hybrid pretreatment of NH and NM on production of xylose and glucose from napier grass

The experimental result from 17 runs on factors NaOH and heat treatment combination in the range 0.1 to 5% NaOH and 60 to 100°C heat treatment gave low yield of fermentable sugars ranging from 0 to 0.3695g/l for xylose and glucose production. A similar result trend with low production of fermentable sugars was observed with NaOH and microwave treatment combination in the range 0.1 to 5% NaOH and 500 to 1000 W microwave intensity. A maximum glucose and xylose production of 0.413g/l and 0.0475g/l respectively was observed. These low productions of fermentable sugars can be attributed to the short pretreatment durations applied in this study

(maximum 3 hours) and low alkaline concentrations (maximum 5% NaOH). These findings differ from the report of Liong *et al.* (2012) who observed glucose yield of 7.3g/l from napier grass pretreated with 7% NaOH for 4 hours at 35°C. Yasuda *et al.* (2013) observed 807mg xylose yield when 100g napier grass was pretreated with low-moisture anhydrous ammonia (LMAA) at room temperature for 4 weeks. Wongwatanapaiboon *et al.* (2012) reported 4.14g/l total reducing sugar yield after napier grass was treated with 7.5% alkaline peroxide at 35°C for 24 hours. According to Moiser *et al.* (2005), alkaline pretreatment yields high sugar concentrations under long resident time (measured in days) at ambient temperature. It is postulated that long process times are necessary because alkaline causes chemical swelling of cellulose fibers in which saponification (base hydrolysis) reaction occurs thus causing disruption of hemicellulose cross links and increasing porosity of biomass (Ogawa *et al.*, 2008).

3.4.5 Linear effect of parameters on xylose and glucose production in hybrid pretreatments

The xylose and glucose yields from the HH and the HM hybrid pretreatments are shown in tables 3.4 and 3.8 respectively. The xylose and glucose yields were greatly influenced by the pretreatment duration, pretreatment temperature, microwave intensity and acid concentration. The production of fermentable sugars and their ratios (xylose to glucose) in the hybrid pretreatments of napier grass is well sensitive to the concentration of HCl. For example, as shown in HH hybrid pretreatment (Table 3.4 runs 2 and 7), at constant pretreatment temperature (80°C) and treatment duration of 97.50 min, a concentration of 5% HCl gave 14.13g/l xylose and 3.86g/l glucose whereas a concentration of 2.55% HCl gave 10.50g/l xylose and 1.41g/l glucose respectively. The observed xylose to glucose ratios were 7:2 and 7:1 respectively. A similar trend was observed for HM hybrid pretreatment at microwave intensity of 750W and duration of 30 min, as a concentration of 5% and 0.1% HCl gave reducing sugar concentrations 14.67 g/l xylose and 3.39g/l glucose, 0.11g/l xylose and 0.24g/l glucose respectively (Table 3.8 runs 4 and 9). Cui and Shen (2012), reported a total reducing sugar yields of 350mg/g substrate when raw beer lees was treated with 4% HCl compared to 250mg/g substrate when 0.5% HCl was used. Raw beer lees biomass analysis showed significant reduction in hemicellulose content (43.54%) after 0.5% and 4% HCl pretreatment, to 6.08% and 1.72% respectively. Thus it is apparent that, high HCl concentration improves reducing sugar yields through increased hemicellulose hydrolysis rate (Idrees *et al.*, 2013).

The microwave intensity of 1000 W gave 14.5g/l xylose and 3.40g/l glucose whereas at 500 W only 10.9g/l xylose and 2.10g/l glucose were obtained at constant HCl concentration (5%) and pretreatment duration (17.5 min) (Table 3.8, runs 1 and 8). This is attributed to increased microwave energy causing increased interactions between hydrogen bonds in cellulose layers which results in freedom of movement and polarisability of groups within cellulose molecules thus reducing crystallinity of biomass and increasing reducing sugar released (Paul *et al.*, 2014). Similarly, Chartchalerm *et al.* (2007) reported gradual increase in densities of wheat straw and barley straw when the microwave intensity was increased. This implies that, a high microwave intensity disintegrates lignocellulosic biomass and dissolves components. At a constant HCl concentration of 5% HCl and pretreatment duration 97.50 min, a high temperature (100°C) gave a yield of 6.61g/l and 4.30g/l of xylose glucose respectively while low temperature (60°C) gave 2.17g/l and 0.662g/l xylose and glucose yields respectively. A xylose to glucose ratio of 3:2 was obtained at 100°C compared to a 3.1 ratio at 60°C (Table 3.4, runs 3 and 8). Idrees *et al.* (2013) reported that low temperature pretreated biomass had a high hemicellulose content and low total reducing sugars while high temperature pretreated biomass had low hemicellulose content and high total reducing sugars. At constant HCl concentration of 5% and pretreatment temperature of 80°C, a long pretreatment duration of 180 min gave high xylose and glucose yields (14.6g/l and 3.8g/l respectively) compared to a short pretreatment duration of 15 min, where 1.45g/l and 0.361g/l xylose and glucose yields respectively were observed. A xylose to glucose ratio of 5:1 was obtained at 180 min compared to a 4.1 ratio at 15 min. These findings are similar to observations by Ogawa *et al.* (2008) and Chartchalerm *et al.* (2007). These differences in the observed xylose to glucose ratios evidence that pretreatment duration, pretreatment temperature, microwave intensity and acid concentration influence the pattern of release of these sugars. However, xylose concentration exceeded glucose concentration under the various conditions investigated.

3.4.6 Interaction of experimental variables on xylose and glucose production in HH and HM hybrid pretreatments

The large variation in fermentable sugar yield observed in HH hybrid pretreatment (0.006 to 14.134 g/l and 0.245 to 5.991g/l for xylose and glucose respectively) illustrates the high susceptibility of these processes on the considered input variables (Table 3.4). A similar pattern of

variability was observed in HM hybrid pretreatment giving fermentable sugars in the range of 0.041 to 14.67g/l xylose and 0.122 to 3.47g/l glucose (Table 3.8). Figures 3.1 to 3.5 illustrate the interactive effect of pretreatment duration, pretreatment temperature, microwave intensity and acid concentration on xylose and glucose production. As shown in figure 3.1a and b, the interactive effect of HCl concentration and pretreatment duration on xylose production has a concave shape indicating that optimum set points were within the design boundaries. A peak of xylose yield about 10g/l was observed within the ranges 2.90 to 3.60% HCl and 147 to 180 min of HCl concentration and pretreatment duration respectively (figure 3.1a). In figure 3.1b, a peak production of about 3g/l glucose was observed between 2.90 to 4.30% HCl and 114.0 to 180 min. The interaction between high acid concentration and long pretreatment duration promoted hemicellulose hydrolysis and degradation of amorphous cellulose thus causing high release of reducing sugars as earlier suggested by Wyman *et al.* (1992) and Yang *et al.* (2010).

As shown in figure 3.2 a, if the pretreatment temperature was maintained at 92°C, an increase of HCl concentration from 0.80 to 4.30% would result in gradual increase in xylose yield from 5g/l to about 11g/l. Similarly in figure 3.2b if the pretreatment temperature was maintained at 92°C, an increase of HCl concentration from 0.80 to 4.30% would result in gradual increase of glucose production from 1g/l to about 4g/l. These findings are consistent with the reported work of Ogawa *et al.* (2008) and Dagnino *et al.* (2013) where high acid concentration and high temperature combination produced better hydrolysis in the pretreatment of water hyacinth biomass and rice hulls respectively. As shown in figure 3.3a and b, a gradual increase in xylose and glucose production from 5 to about 10g/l and from 1 to 3 g/l respectively, was observed when the treatment time was maintain at 114 min and the treatment temperature was gradually increased from 76 to 92°C.

The synergistic effect of HCl concentration and pretreatment duration on the sugars release as illustrated in figure 3.4 a and b, showed that when the HCl concentration was maintained at 5%, a gradual increase in xylose and glucose production from 5 to about 15g/l and from 1 to about 4g/l respectively was observed when the pretreatment time was gradually increased from 10 to 25 min. It is assumed that high acid concentration and long pretreatment duration increase crystalline domain size of lignocellulosic biomass and fibrils by dehydration thus causing hydrophobic lignin to form aggregates which results in amorphous cellulose degradation (Dagnino *et al.*, 2013). As shown in figure 3.5a and b, a gradual increase in xylose from 5 to

about 15g/l and glucose from 1 to 2g/l was observed when HCl concentration was increased from 2.0 to 4.30% with the microwave intensity maintained at 900W. When the microwave intensity was maintained at 500 W, an increase in pretreatment duration from 20 to 30 min gave a sharp increase in xylose and glucose production from 5 to about 15g/l and from 1 to 3g/l respectively (figure 3.5a and b).

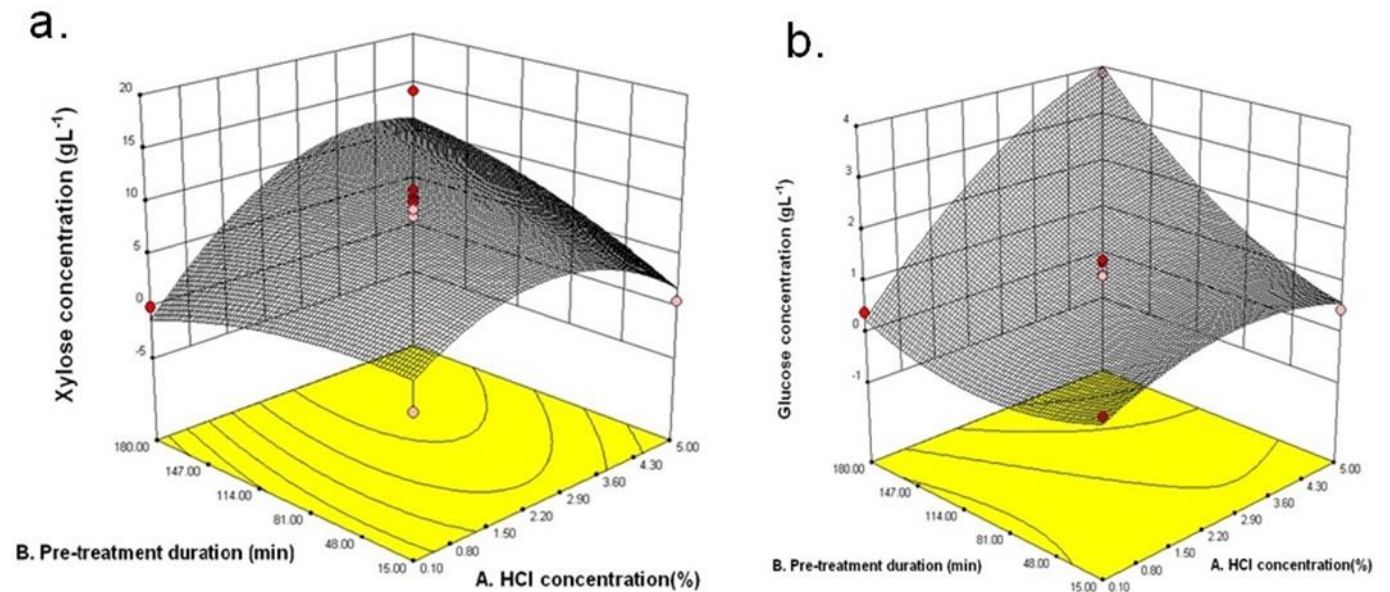


Figure 3.1 - Three dimensional response surface graph showing the interaction of pretreatment duration and HCl concentration on a. xylose production and b. glucose production using HH hybrid pretreatment

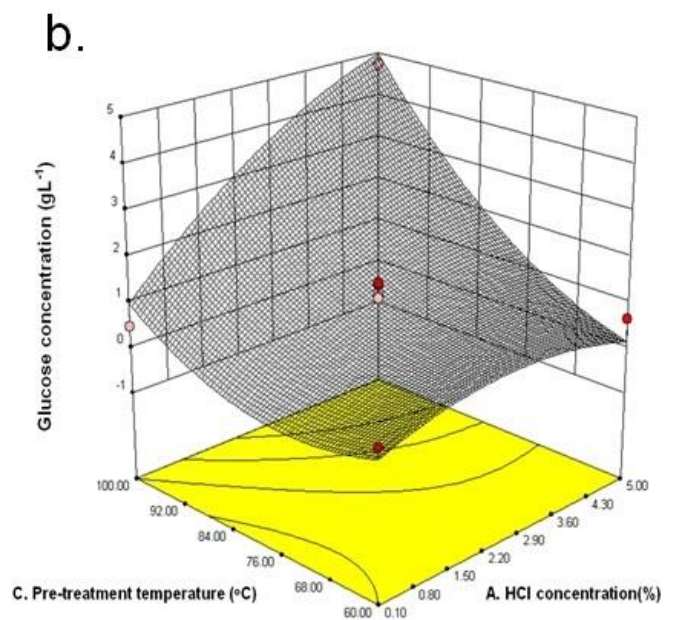
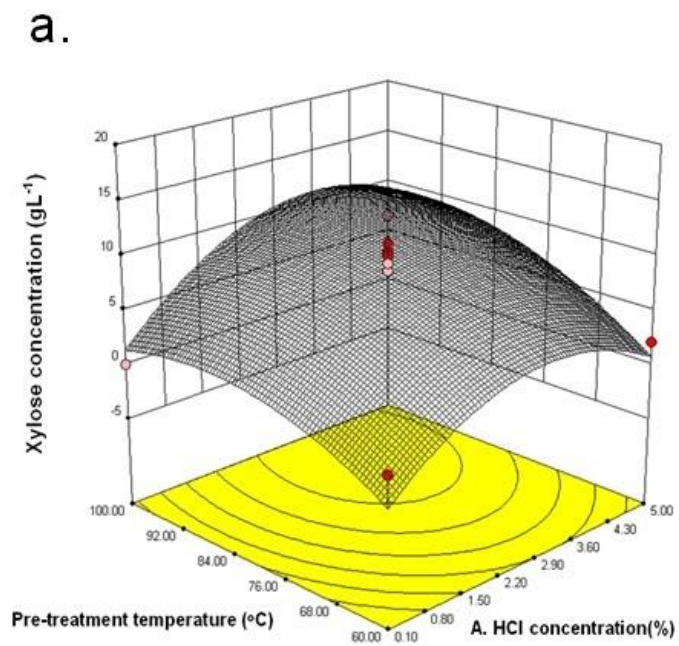
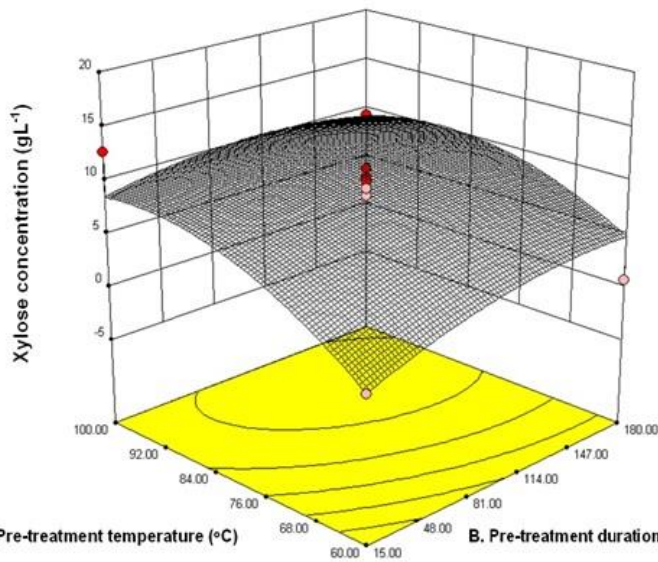


Figure 3.2 -Three dimensional response surface graph showing the interaction of pretreatment temperature and HCl concentration on a. xylose production and b. glucose production using HH hybrid pretreatment

a.



b.

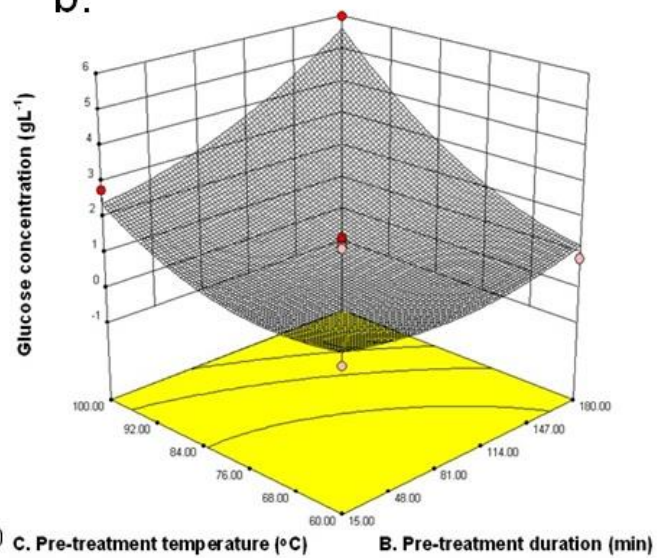


Figure 3.3. Three dimensional response surface graph showing the interaction of pretreatment temperature and pretreatment duration on a. xylose production and b. glucose production using HH hybrid pretreatment

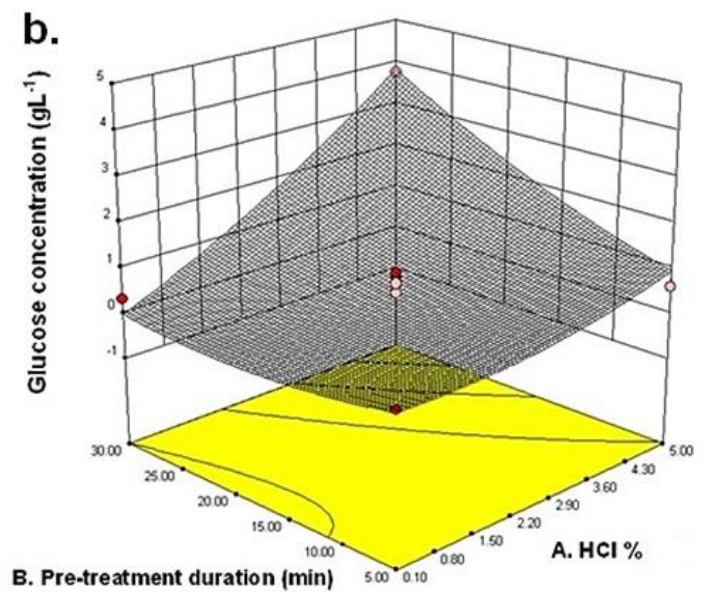
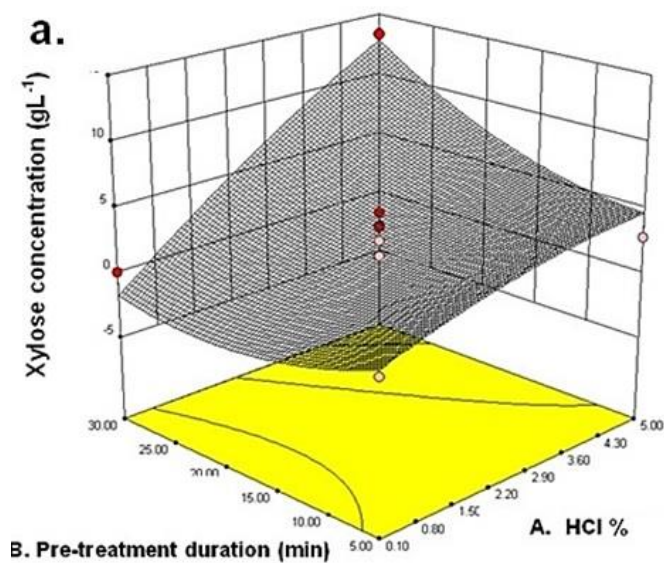


Figure 3.4. Three dimensional response surface graph showing the interaction of pretreatment duration and HCl concentration on a. xylose production and b. glucose production using HH hybrid pretreatment

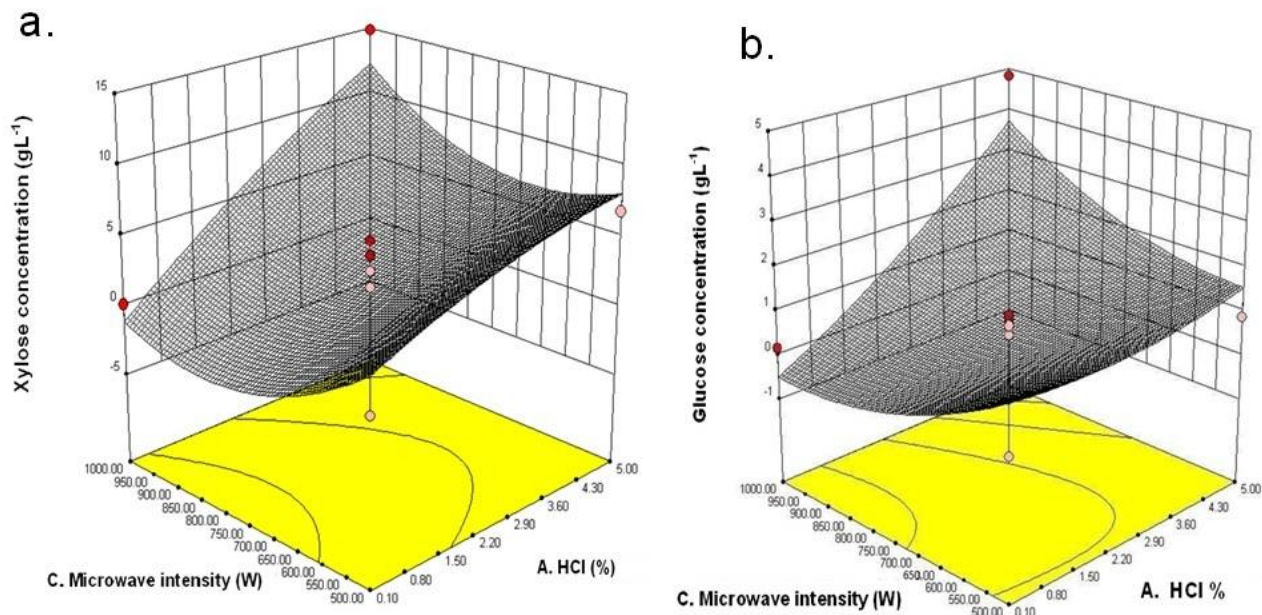


Figure 3.5 -Three dimensional response surface graph showing the interaction of microwave intensity and HCl concentration on a. xylose production and b. glucose production using HH hybrid pretreatment.

3.4.7 Optimization of napier grass pretreatment using hybrid techniques of HH and HM on xylose and glucose production

The obtained optimum operational set points for HH hybrid pretreatment were 4.39% HCl, 180 min at 93.07°C predicting yields of 12.69g/l xylose and 5.99g/l glucose with a xylose to glucose ratio of 2:1. The experimental validations gave 12.83g/l xylose and 2.28g/l glucose thus a 6:1 xylose to glucose ratio. For HM pretreatment hybrid, optimum set points predicted by the model were 5% HCl, 30 min pretreatment duration and microwave intensity of 500W with predicted yields of 18.1g/l and 4.43g/l xylose and glucose respectively with a xylose to glucose ratio of 4:1. Experimental validation gave 15.06g/l and 2.44g/l of Xylose and glucose yields respectively, thus a 6:1 xylose to glucose ratio. These results showed an improvement on earlier report by Yasuda *et al.* (2013), where 0.032g/l and 0.150g/l glucose and xylose production respectively was observed when napier grass was treated with low moisture anhydrous ammonia (LMAA). The observed ratios of xylose to glucose were similar for both hybrids pretreatments.

3.4.8 Preliminary assessment of the optimally pretreated napier for fermentative biohydrogen production

The biohydrogen production experiment was carried out in duplicates using hydrolyzed napier grass substrate pretreated with the earlier optimized HH and HM hybrid methods. For HH and HM pretreated napier grass, the cumulative hydrogen gas volume of 8.57ml and 25.06 ml respectively were observed. Optimization of this process on key physico-chemical variables such as organic loading rate, hydraulic retention time, operational temperature and pH can significantly improve the hydrogen yield. These findings are of special interest for biohydrogen production scale up since this raw material is sustainable and abundantly available and it is regarded as waste.

3.5 Conclusion

The modeling and optimization of set points of hybrid pretreatment techniques for maximum xylose and glucose production from napier grass was evaluated. The findings indicate that fermentable xylose and glucose production from napier grass can be enhanced by combining pretreatments of HCl and moist heat at 4.39% HCl, 93.07°C for 180 min or using a combination of microwave and HCl at 500 W, 5% HCl for 30 min. Additionally, during the hydrolysis of napier grass with the optimized hybrid pretreatments, the ratio of xylose to glucose remained relatively similar (6:1). The optimum generation of xylose and glucose from napier grass significantly leverages its potential as substrate for the production of renewable biomaterials and biofuels such as biohydrogen.

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CHAPTER 4

Modeling and optimization of biohydrogen production from napier grass (*Pennisetum purpureum*) using immobilized mixed microbial consortia – Preliminary scale up

4.1 Abstract

The modeling and optimization of biohydrogen production from pretreated napier grass using immobilized mixed microbial consortia is reported. The optimum set points of substrate concentration and Hydraulic Retention Time (HRT) were investigated using the Response Surface Methodology (RSM). A coefficient of determination (R^2) of 0.79 was obtained and the optimum operational conditions of 19.05% and 139.97 hours for substrate concentration and HRT respectively were obtained, predicting hydrogen yield of 5.31ml H₂/g napier grass. Model validation gave 6.61ml H₂/g napier grass. A semi-pilot scale biohydrogen production in a 13L bioreactor using pretreated napier grass and immobilized mixed microbial consortia was carried out under the optimum operational conditions. A maximum hydrogen fraction of 28.52% and hydrogen yield of 14.03ml H₂/g napier grass was observed at pH 6.3, temperature 37°C after 62 hours of fermentation. This optimum generation of biohydrogen using renewable napier grass highlights potential application of this feedstock towards large scale development of an economical and sustainable hydrogen economy. Additionally, dark fermentative hydrogen production from napier grass using immobilized microbial consortia combines a cheap hydrogen production method with high unit volumetric production rate which further substantiates practicability of economical commercial scale hydrogen production.

Keywords: Biohydrogen production, Immobilized beads, Napier grass, Semi-pilot scale, Modeling, Optimization.

4.2 Introduction

The current heavy reliance on fossil fuels as a primary energy source has led to lamentable problems (Ramprakash and Muthukumar, 2009). Fossil fuels combustion has contributed significantly to greenhouse gasses emissions causing environmental pollution and global warming (Azwar *et al.*, 2014). Hydrogen has attracted increasing global attention as an alternative to conventional fossil fuels because it has high energy yield (142 kJ/g), renewable and non-polluting in nature (Das and Veziroglu, 2001). Commercial scale hydrogen production is costly (Chong *et al.*, 2009). The use of cheap substrates and renewable substrates could significantly lower the hydrogen production cost thus making its production economically feasible at industrial scale.

Lignocellulosic substrates are amenable for fermentative hydrogen production as these are abundant, low cost and sustainable biomass feedstocks (Liu, 2011). Among other lignocellulosic substrates, napier grass (*Pennisetum purpureum*) is a promising feedstock because of its high cellulose content, rapid growth, highly invasive nature, high adaptability and high biomass yields (up to 40 metric ton/ha/year) (Liong *et al.*, 2012; Smith *et al.*, 2013). Its high cellulose (28.88%) and hemicellulose (30.61%) content leverages its potential as valuable source of fermentable sugars (Wongwatanapaiboon *et al.*, 2012). It is a C₄ all season grass species that is widely distributed and native to African grass lands (Smith *et al.*, 2013). Globally, Somerville *et al.* (2012) reported annual cumulative dry matter napier grass yields of 85 tons per hectare while Reddy *et al.* (2012) reported an annual cumulative yield of 40 tons per hectare for South Africa alone. Major advantages of napier grass based hydrogen are the local availability of the biomass, renewability and the feasibility of biomass conversion without high capital costs (Hoogwijk *et al.*, 2003).

There is renewed interest to optimize hydrogen production technologies for high production rates, low energy demands, ease in operation and sustainability (Fang *et al.*, 2002). Improving the microbial substrate conversion rate and unit volumetric production rate are considered key aspects to optimization of hydrogen production (Wu *et al.*, 2006; Zhang *et al.*, 2007). Microbial substrate conversion rate improvement can be achieved through the optimization of key operational parameters while production rate through biomass retention such as microbial cells immobilization (Wu *et al.*, 2006).

Cells immobilization to synthetic polymers and supporting carriers has been observed to retain high biomass concentrations compared to suspended cells (Fang *et al.*, 2002). It provides high cell densities and preserve hydrogen generation activity, thus enhancing production efficiency (Wu *et al.*, 2006). Immobilization of microorganisms for hydrogen production in sodium cellulose sulfate macro-capsules has been reported to increased hydrogen production by more than 30% compared to using suspended culture cells (Sheng and Cheng, 2010). Similarly Vincenzini *et al.* (1982) observed a fourfold increase in hydrogen production using immobilized *Rhodopseudomonas sp.* compared to when suspended cells were used. Immobilized bacteria have also been observed to be advantageous over suspended cells because they can be re-used while still maintaining high production efficiency (Han and Shin, 2004). Though studies have shown that mixed microbial consortia immobilization enhances hydrogen yields, immobilized mixed cultures have been rarely reported for hydrogen production from lignocellulosic feedstocks. Dark fermentative hydrogen production from napier grass using immobilized microbial consortia could be a low cost hydrogen production technology with high unit volumetric production rate.

This study models and optimizes biohydrogen production from pretreated napier grass on input parameters of substrate concentration and HRT using immobilized mixed microbial consortia. Furthermore, it investigates a semi-pilot scale biohydrogen production at optimum substrate concentration and HRT.

4.3 Materials and methods

4.3.1 Inoculum preparation

The anaerobic sludge containing mixed hydrogen-producing consortia was obtained from the Darville Wastewater treatment plant (Pietermaritzburg, South Africa). The sludge was transferred to the laboratory and kept at 4°C prior to use. It was heated for 30 minutes at 100°C to deactivate non-endospore forming hydrogen consuming bacteria while preserving the endospore forming hydrogen producing bacteria.

4.3.2 Immobilization of mixed microbial consortia

For the cells immobilization, 3% sodium alginate was prepared and mixed with pretreated sludge in a 1:1 ratio. The mixture was homogenized for 4 minutes at 60 rpm and transferred drop wise into sterile 2 % CaCl_2 using a peristaltic pump (LKB BROMMA 2120 , USA) to form 1.0 to 1.5 mm biocatalyst beads.

4.3.3 Napier grass pretreatment

The napier grass used as a substrate in this study was harvested at 6 months old from Grassland Science tunnels in the University of KwaZulu-Natal, Pietermaritzburg campus, South Africa. It was dried at 60°C for 72 hours and reduced to particle size of 1mm using a centrifugal mill (Retsch ZM-1, Durban South Africa). Varied concentrations of napier grass powder as specified by the design (Table 4.1) were immersed in 100ml dilute HCl, after which the mixture was treated with moist heat in a waterbath under the optimum pretreatment conditions of 93.07°C for 180 minutes previously established (Mafuleka and Gueguim-Kana, 2015).

4.3.4 Experimental design

The RSM Central composite design was used to determine the optimum set points for napier grass concentration and HRT. The independent variables consisted of napier grass concentration and HRT in the ranges 5 to 35% and 23 to 193 hours respectively. A total of thirteen experimental runs (Table 4.1) were generated with varied conditions and the center points were replicated three times.

4.3.5 Fermentation process set up

Fermentation processes were conducted in 250ml flask bioreactors. The flasks were fed with 80ml of pretreated napier grass and supplemented with 100ml inorganic salts medium (g/l): NH_4Cl 0.5; KH_2PO_4 0.5; K_2HPO_4 0.5; NaHCO_3 4.0; $\text{FeCl}_2 \cdot 2\text{H}_2\text{O}$ 0.15; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.085; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.03; H_3BO_3 0.03; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ 0.01; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.03. Each flask was inoculated with 40g microbial beads and flushed with nitrogen gas for 2 minutes to create anaerobic conditions. The initial pH was adjusted to 6.5 and the flasks were incubated at 35°C in a shaking water bath at 100rpm. The HRT was

varied according to the experimental design.

4.3.6 Modeling and optimization of substrate concentration and HRT

The experimental data recorded from the experimental runs (Table 4.1) were used in a multiple regression analysis to generate a quadratic model that relates hydrogen production to substrate concentration and HRT. The model fitness was assessed using the Analysis of Variance (ANOVA). The optimum set points of substrate concentration and HRT for maximum hydrogen generation were obtained by solving the quadratic equation using the method of Myers and Montgomery (1995). The experimental data were used to develop a second-order polynomial model (Equation 11):

$$Y = \alpha_0 + \alpha_1 x_1 + \alpha_2 x_2 + \alpha_{11} x_1^2 + \alpha_{22} x_2^2 + \alpha_{12} x_1 x_2 \quad (11)$$

Where Y represents the response output, α_0 is the intercept, $\alpha_1 x_1$ and $\alpha_2 x_2$ are linear coefficients, $\alpha_{11} x_1^2$ and $\alpha_{22} x_2^2$ are the quadratic coefficients and $\alpha_{12} x_1 x_2$ represents the interactive coefficient.

4.3.7 Analytical procedures

The volume of evolving biogas fractions of hydrogen, carbon dioxide and methane were continuously monitored using the F-Lab Biogas software described by Gueguim Kana *et al.* (2013). The sampling rate was set to 1 min and the sensors used were the BCP-H₂, BCP-CO₂ and BCP-CH₄ sensors (Bluesens GmbH, Germany), all with measuring range of 0 to 100%.

Cumulative biogas volumes was recursively computed by the software using CO₂, H₂ and CH₄ gas fractions and volumes at each sampling interval according to Equation 12

$$V_{Hi} = V_{Hi-1} + C_{Hi}(V_{Gi} - V_{Gi-1}) + V_H(C_{Hi} - C_{Hi-1}) \quad (12)$$

Where V_{Hi} and V_{Hi-1} represent cumulative hydrogen gas volume at current (i) and previous (i-1) time interval, V_{Gi} and V_{Gi-1} represent biogas volumes in current and previous time intervals, C_{Hi} and C_{Hi-1} represent the fraction of hydrogen gas in the headspace of the bioreactor in the current and previous time intervals and V_H represents the total volume of headspace in the bioreactor.

4.3.8 Semi-pilot scale biohydrogen production using the pretreated napier grass

Semi pilot fermentation was conducted in a 13L (Labfors Infors HT bioreactor, Switzerland) bioreactor. The reactor was fed with 2L of the inorganic salt medium, inoculated at 20% (w/v) with microbial beads and fed with 19.05% (800ml) pretreated napier grass to a total working volume of 4L. The pH was controlled at 6.3 using 5M HCl and 5M NaOH, the reactor operational temperature was 37°C and the agitation was maintained at 150rpm. The fermentation medium pH was monitored using a pH sensor (Mettler Toledo GmbH 405-DPAS-SC-K8S/325, Germany). Nitrogen gas was flushed into the bioreactor for 2 minutes through the gas sparger to create anaerobic conditions. The process was conducted for 62 hours.

The xylose and glucose concentrations produced from the pretreated napier grass were determined using a glucose analyzer (Model 2700 select-dual configuration, YSI USA).

4.3.9 Isolation and characterization of hydrogen producing bacteria

The hydrogen-producing bacteria within the bioreactor were plated out on Differential Reinforced Clostridial Agar (DRCA). Samples were drawn out from the bioreactor at the exponential phase of hydrogen production (43 hours) and transferred into 2ml micro-centrifuge tubes which were stored at -4°C. Serial dilutions in the range 10^{-1} to 10^{-6} were prepared and 100µl of the appropriate dilution was inoculated into DRCA using the spread plate technique. The plates were incubated in anaerobic jars (Oxoid Ltd, UK) at 30°C for 3 days.

4.3.10 DNA extraction and 16S rRNA gene sequencing analysis

Five single colonies were randomly selected based on differences in colony morphologies from the DRCA plates and suspended in 50µl TE buffer. The DNA was extracted using the freeze-thaw method: heating for 10 minutes at 95°C followed by 10 minutes in liquid nitrogen. The samples were then centrifuged at 14000g for 10 minutes and 5µl of the supernatant containing the DNA was amplified by PCR using the published eubacteria primer pair Unibac-II-515f (5'-GTGCCAGCAGCCGC-3'forward primer) and Unibac-II-927rP (5'-

CCCGTCAATTYMTTGGAGTT-3' reverse primer) Lieber *et al.* (2002). PCR-amplification of the DNA was carried out using a G-STORM thermal cycler (Vacutec, South Africa) with the following quantities of reagents per 25µl reaction: 0.5µl forward Unibac- II-515f (5'-GTGCCAGCAGCCGC-3') and reverse Unibac-II-927rP (5'-CCCGTCAATTYMTTGGAGTT-3') primers, 12.5µl 2X KAPA2G Hot Start Ready Mix (Kapa Biosystems, South Africa), 5µl DNA template and 6.5µl nuclease free water. Parameters used for PCR were as follows: an initial denaturing cycle at 94°C for 4 minutes, followed by 35 cycles of 94°C for 20 seconds, 53°C for 30 seconds, 72°C for 60 seconds and a final extension step of 72°C for 10 minutes. The amplified products, 8 µl of amplification mix (500bp) were analyzed by gel electrophoresis in a 1% [1x TBE (Tris-borate EDTA) buffer (10mM, pH 8)] agarose gel stained with SYBR green dye and a GeneRuler™ 1kb DNA ladder (Inqaba biotec, South Africa) as a size marker. The PCR products were sequenced using the ABI3130xI Genetic analyzer. The sequences obtained were compared to 16S rRNA gene sequences deposited in GenBank using the NCBI Basic Local Alignment Search Tool (BLAST, <http://www.ncbi.nlm.nih.gov>).

4.4 Results and discussion

4.4.1 Modeling and optimization of substrate concentration and HRT

Experimental data obtained (Table 4.1) were used to fit a quadratic model relating the independent variables of napier grass concentration and HRT to hydrogen production. A coefficient of determination (R^2) of 0.79 was obtained for the developed quadratic model (Table 4.2), thus, the model can account for 79% of the observed variations within the data. The coefficient of estimates are shown in Table 4.3, where A and B are the linear coefficients of substrate concentration and HRT respectively. These coefficients have a direct contribution to the model output (hydrogen). Hence, term B (HRT) with the coefficient estimate of 25.33 has a significant impact on hydrogen compared to terms A, AB, A^2 and B^2 . The high Fisher-Snedecor distribution (F-value) and low probability value (p-values) 5.34 and 0.0243 respectively implies that the model is significant.

The mathematical model can be expressed according to Equation (13):

$$Y = 94.64 - 2.71 A + 25.33 B - 10.84 A B - 44.66 A^2 - 24.75 B^2 \quad (13)$$

Where Y is the H₂ production volume in ml. A and B are linear coefficients of substrate concentration and HRT respectively, AB are interactive coefficients and A² and B² are square coefficients.

Table 4.1. Biohydrogen production from pretreated napier grass under varied substrate concentration and HRT

Run	Substrate (%)	HRT (hours)	hydrogen (ml)
1	30	168	2.57
2	20	192.85	108.99
3	20	23.15	0
4	20	108	102.43
5	20	108	134.19
6	30	48	0
7	20	108	76.32
8	34.14	108	27.65
9	20	108	89.47
10	20	108	70.77
11	10	168	53.41
12	5.86	108	1.71
13	10	48	7.49

Table 4.2: Analysis of variance for biohydrogen production

Source	Sum of Squares	df	Mean Square	F-value	p-value	R²
Model	22068.92	5	4413.78	5.34	0.0243	0.79

df: degrees of freedom, F-value: Fisher-Snedecor distribution value, p-value: probability value, R²: coefficient of determination

Table 4.3: Coefficient of estimate of the model and the confidence intervals for biohydrogen production

Factor	Coefficient estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	94.64	1	12.85	64.24	125.03
A	-2.71	1	10.16	-26.73	21.32
B	25.33	1	10.16	1.3	49.35
AB	-10.84	1	14.37	-44.82	23.14
A²	-44.66	1	10.9	-70.42	-18.89
B²	-24.75	1	10.9	-50.52	1.01

df: degrees of freedom, 95% CI Low: 95% confidence intervals (low limit), 95% CI High: 95% confidence interval (high limit)

4.4.2 Interaction of substrate concentration and HRT on biohydrogen production

Table 4.1 illustrates the hydrogen production from the varied substrate concentration and HRT. The hydrogen production obtained from the varied substrate concentrations and HRT ranged from 0 to 134.19 ml (Table 4.1). This wide range indicates the sensitivity of biohydrogen fermentation to substrate concentration and HRT. Figures 4.1 and 4.2 show the three dimensional response surface and contour map plot respectively of substrate concentration and HRT interaction on biohydrogen production. In figure 4.1, the interactive effect of these parameters on hydrogen production showed a concave shape which indicates that their optimum set points were within the boundaries of the search range. A peak hydrogen volume of 100 ml was observed within the ranges 15-20% and 138-168hours of substrate concentration and HRT respectively. Fan *et al.* (2006), noticed significant increase in hydrogen yields with increasing substrate concentration from 5 to 20g/l. With an HRT of 108 hours, an increase in substrate concentration from 25 to 30% caused a decrease in hydrogen production from 80 to 20 ml (as shown in figures 4.1 and 4.2). The decrease in hydrogen production was presumably due to the accumulation of volatile fatty acids (VFAs). These have been reported to occur at very high substrate

concentration and are believed to inhibit the hydrogenase activity (Fan *et al.*, 2006).

With a constant substrate concentration of 20%, a gradual increase in HRT from 78 to 138 hours showed a linear increase in hydrogen production from 60 to 100ml (figures 4.1 and 4.2). A further increase of HRT above 138 hours resulted in a gradual decrease in hydrogen production from 100 to 80 ml (as shown in figures 4.1 and 4.2). Liu *et al.* (2011); Zhang *et al.* (2007) and Thanwised *et al.* (2012) reported similar optimum HRT's ranging from 100 to 144 hours as ideal for hydrogen production with yields of 21ml H₂/g TVS, 883.19 H₂/L/ d and 1.6 mol H₂/g glucose observed respectively.

4.4.3 Optimization of biohydrogen production using the central composite design

Optimization for biohydrogen production suggested operational set points of 19.05% and 139.97 hours substrate concentration and HRT respectively with a predicted hydrogen production of 101ml thus a yield of 5.31ml H₂/g napier grass. After model validation hydrogen production of 117ml was obtained thus a yield of 6.61ml H₂/g napier grass.

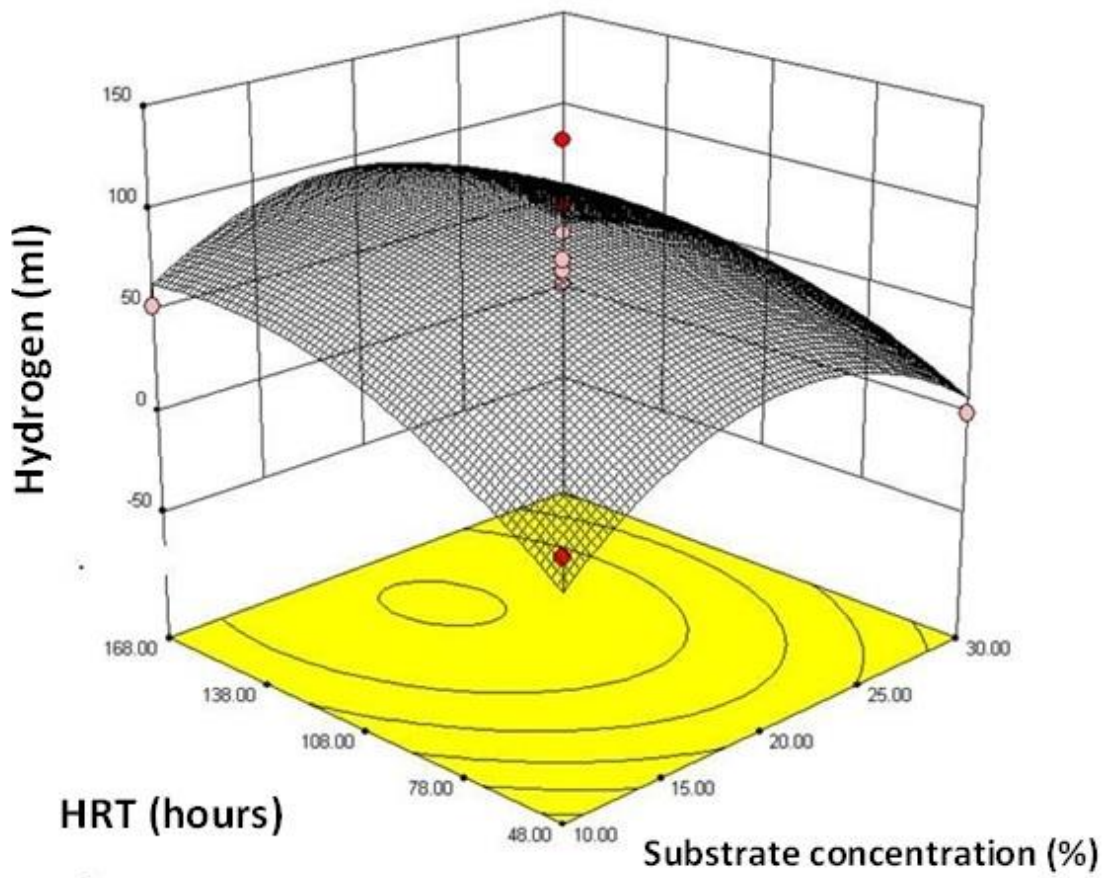


Figure 4.1 - Three dimensional response surface curve of substrate concentration and HRT interaction on biohydrogen production.

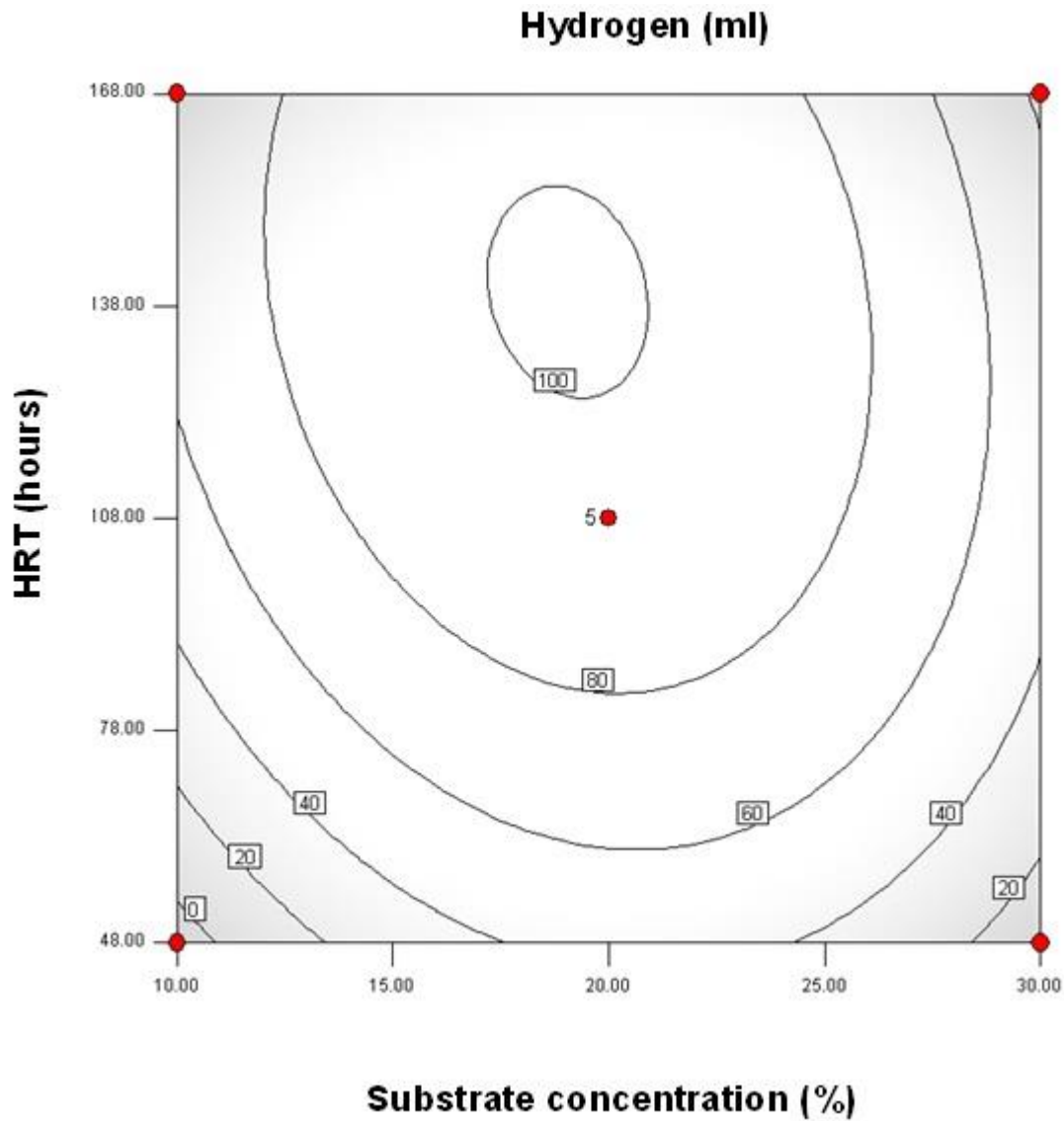


Figure 4.2 - Contour map plot of substrate concentration and HRT interaction on biohydrogen production

4.4.4 Semi-pilot scale biohydrogen production

4.4.4.1 Hydrogen production phases

Figure 4.3 a and b shows the biogas evolution and sugar consumption over the fermentation duration at semi-pilot scale biohydrogen production. Hydrogen production started after 24 hours of fermentation (figure 4.3 a). This long lag phase could be attributed to the slow uptake

of sugar and nutrients by the immobilized hydrogen producing bacteria since immobilization of microorganisms into synthetic polymers has been observed to alter spatial organizations at molecular level thus slowing down microbial metabolism (Kraemer and Bagley, 2007). As shown in figure 4.3, xylose and glucose concentrations remained constant during the first 24 hours of fermentation. Papadikis *et al.* (2010) augmented the retention time from 4 to 24 hours to increase immobilized cell permeability thus enhancing the uptake of nutrients and substrates into microbial cells. A similar lag phase duration of 21 hours has been reported by Lee and Chang (2010) using cellulosic food waste in a two-stage pilot scale process for biohydrogen production.

Figure 4.4 shows cumulative biogas evolution during the fermentation process. Exponential phase of hydrogen production was observed from 24 hours to 60 hours with a maximum hydrogen fraction of 28.52% and cumulative volume of 596.09ml thus a maximum hydrogen yield of 14.03ml H₂/g napier grass at 44 hours (figures 4.3 a and 4.4). This lengthy exponential phase (36 hours) is characteristic of immobilized inoculum. Vasavi *et al.* (2014) reported a 5 fold increase in hydrogen production from immobilized *Rhodopseudomonas rubra* compared to suspended cells using glucose as a carbon source. The authors observed retention of exponential phase for 24 hours using immobilized cells compared to 6 hours using suspended cells. The retention of exponential biohydrogen production phase may be due to high biomass retention, even distribution of biomass throughout the reactor volume and high substrate conversion rates in the immobilization matrices (Kumar and Das, 2001; Hu *et al.*, 2007).

An increase in hydrogen fraction corresponded to a decrease in xylose and glucose concentrations (from 24 to 60 hours) (figure 3.4 a and b). Xylose and glucose concentrations decreased from 7.13 to 0.005g/l and 3.02 to 0.011g/l respectively, which indicates that xylose and glucose utilization by the immobilized consortia were channeled for hydrogen production. Carbohydrates are essential substrates and can also be a limiting factor for hydrogen producing microorganisms affecting both cell growth and hydrogen production (Fabiano and Perego, 2012). Hydrogen is produced in the acidogenic phase through the hydrolysis of carbohydrates via either acetate or butyrate fermentation reaction (Khanal *et al.*, 2004; Hung *et al.*, 2007).

The lengthy exponential phase could also be attributed to pH control of the fermentation medium at 6.3 (Hung *et al.*, 2007). The control of pH at optimum set point balances the uptake of protons by hydrogenases thus preventing metabolic shifts and suppresses hydrogen consumption by the

hydrogen consuming bacteria which may be present in the medium (Badiei *et al.*, 2012). A pH value lower than the optimum set point will result to low intracellular ATP concentrations which will limit carbohydrates uptake and cell growth. Meanwhile pH values higher than optimum can inhibit hydrogenase activity thereby causing a metabolic shift from acidogenesis to solventogenesis which will result to low hydrogen production (Sekoai and Gueguim Kana, 2014).

A decline in hydrogen production was observed from 60 hours to 62 hours with a minimum hydrogen fraction of 24.46% (figure 4.3 a). This decline might be due to substrate depletion as the concentration of xylose and glucose decreased to 0g/l after 60 hours of fermentation (figure 4.3 b). The depletion of the carbohydrates might have led to a metabolic switch from acidogenic to solventogenic process thus forming products like acetone, butanol and ethanol instead of hydrogen (Sekoai and Gueguim Kana, 2014). Accumulation of these metabolites has been reported to inhibit hydrogen production (Wu *et al.*, 2006).

4.4.4.2 Carbon dioxide evolution

Carbon dioxide production started from 24 hours, and reached a peak fraction of 43.25% and a cumulative volume of 1578.52ml at 41 hours (figures 4.3 a and 4.4). A steady decline in the carbon dioxide fraction (from 43.25% to 1.07%) was observed from 35 hours to 42 hours of fermentation. A high correlation coefficient of 0.97 was observed between carbon dioxide and hydrogen evolution at exponential phase from 35 to 42 hours of fermentation (figure 4.3a). It is likely that butyrate fermentation which is the first reaction in the sequence of reactions for hydrogen production was thermodynamically favorable at 30 hours where peak carbon dioxide fraction of 38% is observed. This pathway has high CO₂ to H₂ theoretical yield which is 2mol CO₂/mol glucose (Thauer *et al.*, 1977). After 40 hours, acetate fermentation might have become thermodynamically favorable, this assumption is due to higher H₂ to CO₂ fraction. Acetate fermentation has a high H₂ to CO₂ theoretical yield of 4 H₂/mol glucose (Thauer *et al.*, 1977).

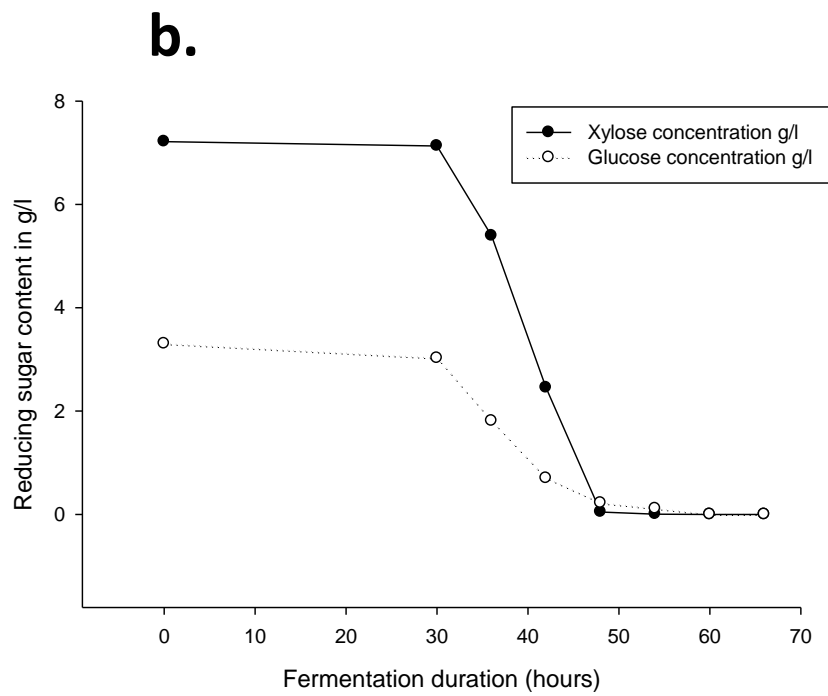
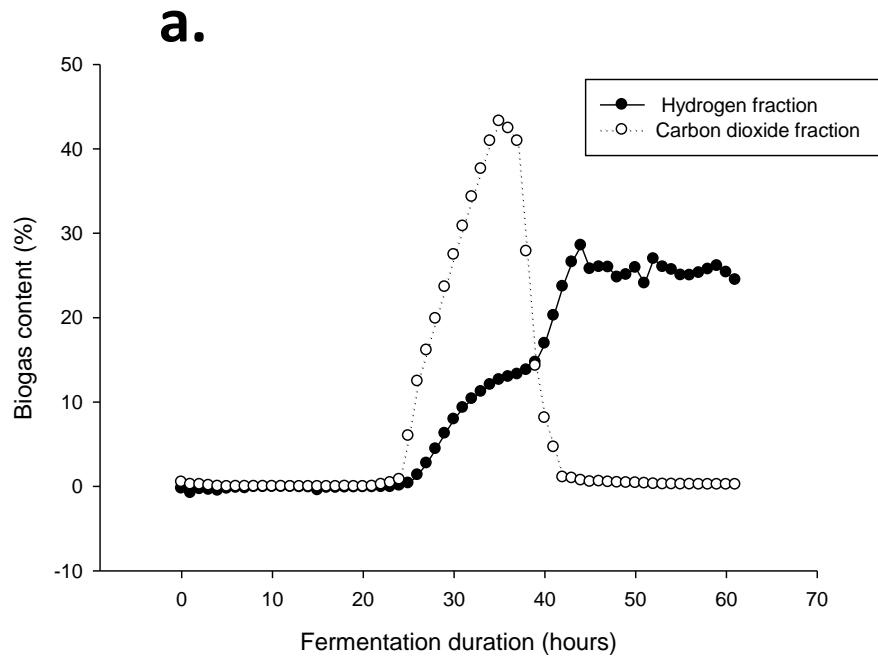


Figure 4.3 – Time course of a. biogas evolution and b. sugar utilization during the semi-pilot scale hydrogen production

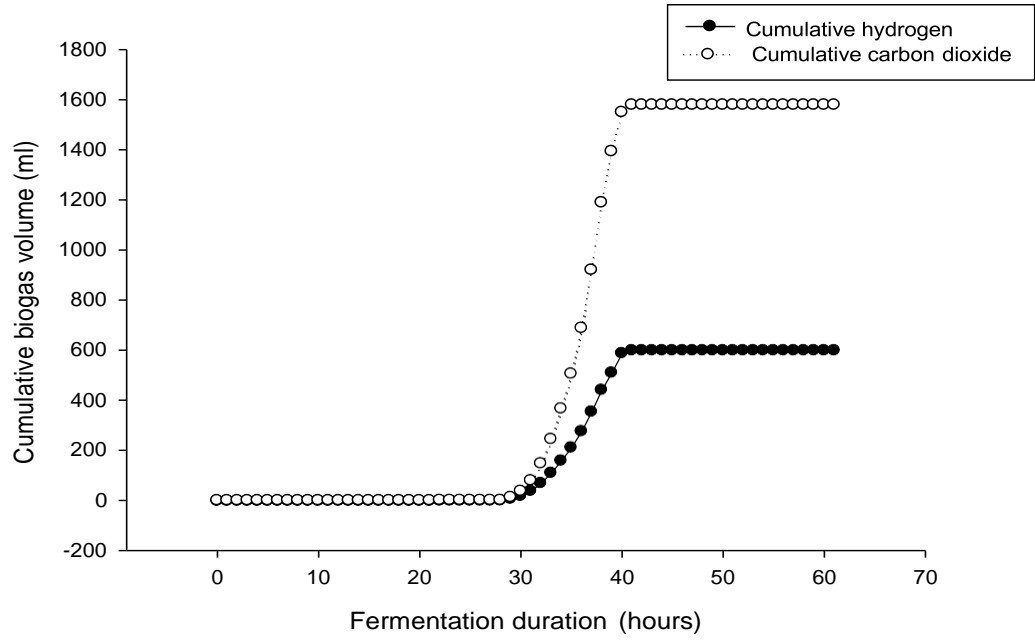


Figure 4.4 – Cumulative biogas evolution during fermentation process

4.4.5 Isolation and characterization of hydrogen producing bacteria

PCR profile of hydrogen producing bacteria is presented in figure 4.5. In lanes 1 to 15, the PCR amplicons of bacteria grown on DRCA can be observed as major bands. Studies on microbial characterization of hydrogen producers in digested sludge use DRCA for the cultivation and enrichment of spore-forming *Clostridium* species (Eigruber and Reuter, 1995; Byrne *et al.*, 2008; Liu and Tay, 2002).

The sequences obtained for the isolates showed high similarity of 99, 98, 98, 98 and 98% to *Enterobacter aerogenes*, *Streptococcus thermophilus*, *Streptococcus thermophilus*, *Enterobacter aerogenes* and *Streptococcus thermophilus* respectively (shown in Table 4.4). Ma *et al.* (2012) also reported the presence of *Enterobacter aerogenes* in the study of hydrogen producing sludge. Facultative anaerobe *E. aerogenes* metabolizes carbohydrates through the Embden-Meyerhoff pathway for hydrogen production (Tanisho and Ishiwata, 1995). *Enterobacter* species are known to produce carbon dioxide and hydrogen in the ratio 2:1 at 37°C (Rachman *et al.*, 1998). Kim *et al.* (2006) observed non-spore-forming *Enterobacter* species after heat shock pretreatment of hydrogen producing sludge. Survival of these bacterial species might be due to the solid matter in the sludge which has been observed to harbor vegetative cells and non-endospore-formers thus enabling most bacterial species to survive high temperatures (Lu *et al.*, 2009).

The absence of *Clostridia* growth could be attributed to possible presence of oxygen during cultivation. It has been observed that smallest quantities of oxygen inhibit the growth of this bacterium specie through lowering the adenylate charge thus causing cell death. Studies suggest the use of anaerobic chambers for the inoculation and cultivation of these species (Hung *et al.*, 2011). This could also be due to the short incubation time of 3 days which might have led to growth of the facultative anaerobic and fast growing bacterial strains outcompeting fastidious strict anaerobic *Clostridia* (Badiei *et al.*, 2012). Badiei *et al.* (2012), analyzed the microbial community of hydrogen producing sludge after heat shock pretreatment at 85°C for 60 minutes using DGGE. Their findings revealed 50% isolates were members of the *Streptococci* species, 30% were *Lactobacillus* species and only 20% were *Clostridium* species. The authors presumed that *Clostridium* species were either present as spores or the active cells were too small to be detected on DGGE.

Although *Streptococci* species have been identified in hydrogen producing sludge, their role in

hydrogen production is still unclear (Liu and Tay, 2002; Hung *et al.*, 2011). It is believed that these microbial species help in aggregation of hydrogen producing microorganisms. *Streptococcus* species produce extracellular polysaccharides which promote granulation by agglomeration of bacterial cells thus enabling the population to withstand heat shock and long HRT's, therefore can survive in the reactor (Hung *et al.*, 2011). Chu *et al.* (2011) reported that the presence of *Streptococci* species also helps maintain high hydrogen yielding microorganisms in the net like biological granules. *Streptococci* species are facultative anaerobic microorganisms that can tolerate high NaCl concentration (Lavilla Lerma *et al.*, 2014). These microbial species are found in gastrointestinal tract of humans, soil and in waste and can tolerate temperatures up to 65°C (Lavilla Lerma *et al.*, 2014).

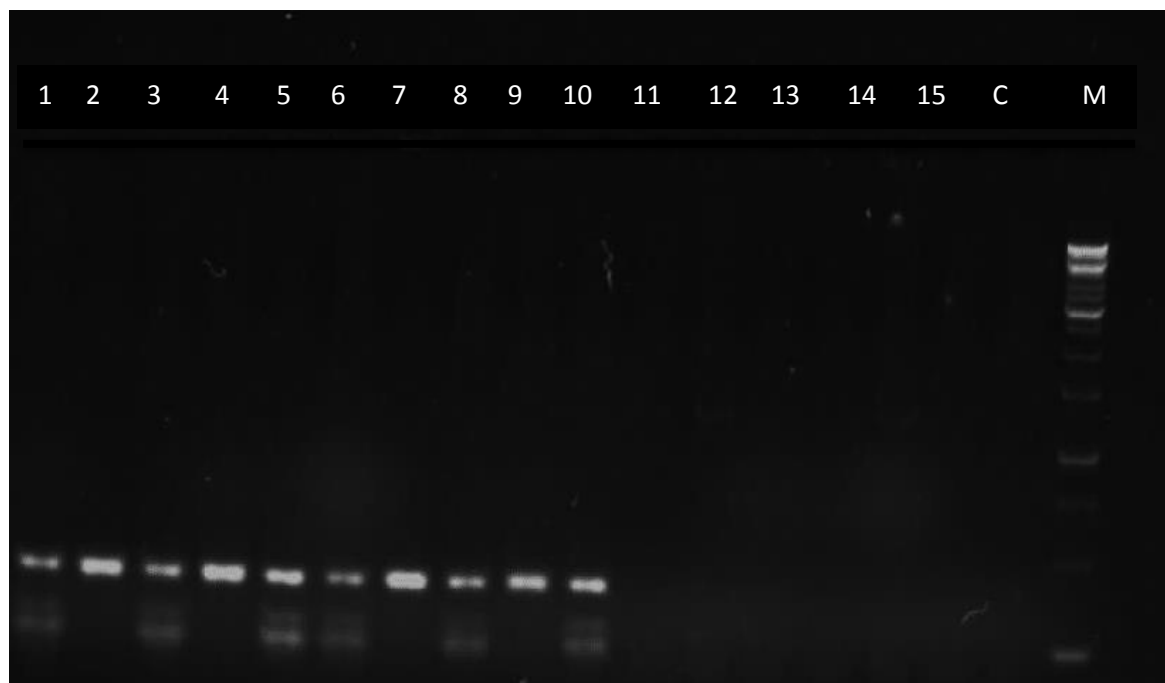


Figure 4.5 - PCR profile of hydrogen producing bacteria: Lanes 1 to 15 represents the PCR products of bacteria grown in DRCA and C represents the non-template control. A 1kb DNA Ladder (M) GeneRuler™ was used in a 1% (w/v) agarose gel to determine the sizes of the isolated DNA fragments (500bp).

Table 4.4-Affiliation of isolates to published species using 16S rRNA gene sequences

Isolates	Organism affiliation	NCBI blast results	
		Accession number	Identity (%)
no. 1	<i>Enterobacter aerogenes</i>	NC 015663.1	99
no. 2	<i>Streptococcus thermophilus</i>	NC 006449.1	98
no. 3	<i>Streptococcus thermophilus</i>	NC 006449.1	98
no. 4	<i>Enterobacter aerogenes</i>	NC 015663.1	96
no. 5	<i>Streptococcus thermophilus</i>	NC 006449.1	98

4.5 Conclusion

The modeling and optimization of biohydrogen production from pretreated napier grass using immobilized mixed microbial consortia was conducted. The study revealed optimum operational set points of 19.05% and 139.97 hours for substrate concentration and HRT respectively with predicted hydrogen yield of 5.31ml H₂/g napier grass. Model validation gave 6.61ml H₂/g napier grass. Furthermore, the hydrogen production at semi-pilot scale showed an exponential phase of 36 hours, with a peak hydrogen fraction of 28.52%, cumulative volume of 596.09ml and hydrogen yield of 14.03ml H₂/g napier grass. The dark fermentative hydrogen production from napier grass which is considered as waste, using immobilized consortia combines a low cost hydrogen production technology and high production rate, thus positively impacting the bioprocess economics.

4.6 References

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CHAPTER 5

Conclusions and recommendations

5.1 Conclusions

In this study, napier grass was used as a feedstock for fermentative hydrogen production. Hybrid pretreatment techniques for optimum xylose and glucose production from napier grass namely HCl and moist heat (HH), HCl and microwave (HM), NaOH and moist heat (NH) and NaOH and microwave (NM) were modeled and optimized. The potential of pretreated napier grass for fermentative biohydrogen production using immobilized microbial consortia was reported. Based on the experimental findings, the following conclusions can be made:

- 5.1.1 Xylose and glucose production can be enhanced by an optimal combination of HCl and moist heat at 4.39% HCl, 93.07°C for 180min, or by HCl and microwave at 5% HCl, 500W for 30min. Optimum xylose and glucose concentrations of 12.83g/l and 2.28g/l were observed under optimized HH pretreatment and 15.06g/l and 2.44g/l were observed under optimized HM pretreatment conditions. These findings demonstrate that napier grass is a source of fermentable sugars that can be channeled for biofuel and biomaterial production. Moreover, HH and HM hybrid pretreatment strategies were observed efficient and effective napier grass pretreatment approaches.
- 5.1.2 A maximum hydrogen fraction of 6.61ml H₂/g napier grass was achieved using pretreated napier grass at optimum set points 19.05% and 139.97 hours for substrate concentration and HRT. These findings highlight that physico-chemical parameter optimization is critical for biohydrogen process development.
- 5.1.3 The feasibility of biohydrogen production from pretreated napier grass using immobilized mixed microbial consortia at semi-pilot scale was evaluated. A peak hydrogen fraction of 28.52% and hydrogen yield of 14.03ml H₂/g napier grass was achieved at pH 6.3, temperature 37°C after 62 hours of fermentation. These findings demonstrated fermentative hydrogen production from napier grass using immobilized beads, a promising approach to cheap and sustainable hydrogen economy.

5.2 Recommendations for future work

For successful application of napier grass for biohydrogen production processes, the following recommendations are suggested for future work:

- 5.2.1 Napier grass utilization will markedly improve biohydrogen production process since the feedstock is abundant, sustainable and has high cellulose content.
- 5.2.2 Studies should explore pretreatment hybridization by combining other existing pretreatment methods for efficient and economical lignocellulosic biomass pretreatment and optimum fermentable sugar generation.
- 5.2.3 Novel bioreactor configurations coupled with real time monitoring systems in preliminary screening experiments for identification of independent parameters should be considered to determine suitable major parameters and set points. This will allow suitable parameter range selection which will translate to successful biohydrogen optimization.
- 5.2.4 Characterization of hydrogen producing and consuming microorganisms present in the sludge at molecular level will aid on more insightful understanding of hydrogen production pathways. This understanding will help in suppressing the hydrogen consumption pathways, sustain and optimize the hydrogen production rates. Studies should focus on microbial genetic engineering tools to manipulate these pathways.

Appendix A: Publication