Evaluation of biohydrogen production potential of sugarcane bagasse using activated sludge in a dark fermentation process

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Karen Reddy

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DECLARATION

Evaluation of biohydrogen production potential of sugarcane bagasse using activated sludge in a dark fermentation process

Karen Reddy

I hereby declare that the dissertation represents my own work. It has not been submitted before for any diploma/degree or examination at any other Technikon/University.

[redacted]

Karen Reddy

22/08/16

Date

2016

Reference Declaration in Respect of a Master's Dissertation

I, Karen Reddy and Professor Faizal Bux do hereby declare that in respect of the following dissertation:

Evaluation of biohydrogen production potential of sugarcane bagasse using activated sludge in a dark fermentation process

As far as we know and can ascertain: no other similar dissertation exists: all references as detailed in the dissertation are complete in terms of all personal communications engaged in and published works consulted.

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APPROVAL

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This 22 day of August 2016, at the Durban University of Technology

ABSTRACT

Anaerobic dark fermentation is an efficient biological process to produce hydrogen from waste material. In South Africa, this technology has not been explored adequately to extract energy from biological wastes. Within the KwaZulu Natal region of South Africa, the sugar industry is a prominent venture that produces mass quantities of sugarcane bagasse amongst other waste products. This by-product can be an ideal source of substrate for biohydrogen generation. In this study, sugarcane bagasse was used as the main substrate for biohydrogen production by anaerobic fermentation using sewage sludge as the inoculum. Different pre-treatment methods were employed to maximize the release of fermentable sugars from the lignocellulosic biomass. Among the different pre-treatment methods employed, the maximum sugar yield (294.4 mg/g) was achieved with 0.25% H2SO4 for 60 minutes at 121°C. Prior to inoculation, the sewage sludge was also subjected to thermal pre-treatment to eliminate methanogens. Thermal pre-treatment of inoculum sludge for 30 min was effective in eliminating methanogens. Fluorescence in situ hybridization was used to positively identify the hydrogen producing bacteria present before and after treatment. The pre-treated substrate and inoculum was integrated into a dark fermentation process to further optimize the effect of pH, substrate to biomass, iron and magnetite nanoparticles on hydrogen production. The maximum hydrogen production (1.2 mol/mol glucose) was achieved at a pH range of 5-6, a substrate to biomass ratio of 3.5, and iron and magnetite nanoparticle concentration of 200 mg/L. Microbial analysis using quantitative polymerase chain reaction has confirmed the dominance of *Clostridium* spp. in the reactor. The highest hydrogenase gene activity (number of copies of hydrogenase gene expression/ng DNA) was recorded in the reactor supplemented with magnetite nanoparticles with lowest being in the raw sludge. There was a direct positive correlation between the hydrogenase gene copy number and the hydrogen yield obtained at different reactor conditions. Scanning electron microscopy was a useful to visually analyse the interaction of microorganisms with activated sludge. This study highlights the significance of anaerobic microorganisms from waste sludge being able to utilize agricultural waste material to produce biohydrogen which could be further scaled up for continuous hydrogen production. In addition, statistical tools used to predict the possible sugar (Design of experiments) and hydrogen yields (Gompertz model) produced would be helpful in saving time during full-scale operation of biohydrogen producing reactors.

PREFACE

The outputs of the Masters qualification include:

Publications

Gupta, S. K., Kumari, S., **Reddy, K**. & F Bux 2013. Trends in biohydrogen production: major challenges and state-of-the-art developments. *Environmental Technology*, 34**,** 1653-1670.

Reddy, K., Nasr, M., Gupta, S. K., Kumar, S., Kumari, S. & F Bux. Biohydrogen production from sugarcane bagasse hydrolysate: effect of pH, S/X, Fe^{2+} and magnetite nanoparticles (Submitted).

Conference submissions and acceptance

Reddy, K., Nasr, M., Gupta, S. K., Kumar, S. & F Bux. 2016. Evaluation and optimization of sugarcane bagasse hydrolysis: a statistical approach. Poster presentation. ECO-BIO 2016 conference, 6-9 March, Rotterdam, Netherlands.

Reddy, K., Nasr, M., Gupta, S. K., Kumar, S. & F Bux. 2014. Evaluation of acid pre-treatment methods for the efficient release of sugars from lignocellulosic bagasse for biohydrogen production. Poster presentation. International Bioenergy Conference, 11-13 March, Manchester, UK.

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LIST OF ABBREVIATIONS

SCB: Sugarcane bagasse

MSY: Mean sugar yield

HPLC: High pressure liquid chromatography

SEM: Scanning electron microscopy

FAM: 5'- end with 6-carboxy-fluorescein

DAPI: 4',6-diamidino-2-phenylindole

FISH: Fluorescent in situ hybridization

WWTP: Wastewater treatment plant

KWTP: Kingsburgh wastewater treatment plant

NWTW: Northern wastewater treatment works

DGGE: Denaturing gradient gel electrophoresis

QPCR: Quantitative polymerase chain reaction

HRT: Hydraulic Retention time

CSTR: Continuous Stirred Tank Reactor

MBR: Membrane Bioreactor

UASBRr: Upflow Anaerobic Sludge Blanket reactor

TS: Total solid

TSS: Total suspended solids

VSS: Volatile suspended solids

COD: Chemical oxygen demand

SHPR: Specific hydrogen production rate

HY: Hydrogen yield

SRT: Sludge retention time

CHAPTER ONE:
GENERAL INTRODUCTION AND OBJECTIVES OF STUDY

The rapid influx in fossil fuel depletion, escalating greenhouse gas emission and the astonishing shifts in typical weather patterns are amongst some of the reasons for the need for exploration of renewable energy sources that will be able to reinstate conventional energy usage in the expectant future. Moreover, conventional energy production methods such as electrochemical and thermochemical methods are highly energy intensive as well as known to pose threats to the environment. The continuous climb in global energy demand has encouraged widespread dependency on non-renewable resources. A major fraction of energy is derived from fossil fuels, thus repetitive use of these resources has brought about the desolation of ascertaining an alternate sustainable energy source. In addition, it conveys ineffaceable distress to the atmosphere. Fossil fuel emission is known to be one of the contributors to global warming. According to previous studies, every year more than a billion tons of carbon dioxide had been released into the atmosphere (Ogbeide, 2010) Therefore, there is a continuous hunt for potential non-carbonaceous alternatives of conventional fossil fuels as an energy source.

Hydrogen is one of the renewable energy ventures that have been recognized as a rescuer fuel of the future. Amongst all gaseous fuels, hydrogen has high energy content compared to other hydrocarbon based fuels (Hasyim *et al.*, 2011). It possesses the ability to serve as a convenient energy carrier that allows energy to remain stored or distributed and consumed. Hydrogen produced can be useful as an energy source for motor vehicles, electricity generation, as a coolant in generators, rocket fuel and as an oxygen forager (Levin, 2004, Nagano *et al.*, 2002). This carbon free energy carrier has qualities which extend publically, environmentally and economically. From these the most significant would be its environmental impact in reducing toxic gas emission which will eventually reduce the rate of global warming. Its non-polluting nature makes this fuel an ideal choice as an alternate energy source (Armor, 1999).

Biological hydrogen production has been considered to be an attractive option and has gained global attention during the last few decades. Biological hydrogen production can be carried out by numerous photosynthetic and fermentative microorganisms (Sen *et al.*, 2008), which includes dark and photo fermentation. Photobiological hydrogen production is based on water

splitting to form oxygen and hydrogen molecules, in the presence of a light source by algae and cyanobacteria (Gimpel *et al.*, 2013). Biological hydrogen production with such photosynthetic microorganisms can occur in closed photobioreactors under certain conditions. For instance, studies have discovered that an algal strain deprived of sulphur, switched from the production of oxygen to the production of hydrogen (Melis and Happe, 2001). The method seems to be a promising, as it requires only water and solar energy to produce hydrogen gas, its downfall lies in light efficiency, cost of energy input and sensitivity of the hydrogenase enzyme to the by-product, oxygen (Oh *et al.*, 2011).

Alternatively, dark fermentative hydrogen production involves the conversion of organic substrates into a mixture of volatile fatty acid products and hydrogen. In the absence of oxygen hydrogen can be produced from a mixed microbial consortium of bacteria. A wide range of waste material can be integrated into this cost effective method of hydrogen production. Currently, the volatile fatty acids produced during fermentation are used in the synthesis of valuable chemicals. These by-products have raised much attention in the biorefinery industry. Additionally, the by-products of dark fermentation (organic acids) can also be used for hydrogen production by combining dark and photo fermentation processes which would improve the overall hydrogen yield (Bastidas-Oyanedel *et al.*, 2015).

This study was directed towards fermentative biological hydrogen production which raises much hope in producing sustainable energy at a low cost due to the prospect of incorporating lignocellulosic agricultural waste material and sewage waste into the production process. Dark fermentative processes involves anaerobic and facultative anaerobic bacteria that ferment organic compounds that thereafter aid in the production of hydrogen and some of the low carbon chain reactions (Kapdan and Kargi, 2006). Agricultural waste such as sugarcane bagasse, molasses and some industrial effluents contain sugars that can be utilized by microorganisms as a feedstock (Saini *et al.*, 2015). This not only benefits in reducing the costs associated with hydrogen production but encourages waste global minimization efforts (Guo *et al.*, 2010). The qualities that hydrogen energy displays makes this technology a more favorable venture for research and development.

AIM AND OBJECTIVES

Aim

To optimise biohydrogen from sugarcane bagasse using activated sludge as inoculum

Objectives

- Evaluation of suitable pre-treatment methods to release fermentable sugars from sugarcane bagasse.

- Optimization of thermal pre-treatment of sludge inoculum to eliminate hydrogen consuming microorganisms.

- Assessment of operational parameters (sugarcane bagasse hydrolysate utilization, pH and iron concentration) on biohydrogen production in an anaerobic dark fermentation process at laboratory scale.

- Ascertain the influence of different concentrations of magnetite nanoparticle on hydrogen production potential.

- Identification and characterization of microbial community structure in treated sludge and during fermentation by molecular techniques.

CHAPTER TWO:
LITERATURE REVIEW

2.1 Hydrogen

Elemental hydrogen is known to be one of the most abundant constituents in the atmosphere and is 1.11g lighter than the mass of air (Dasa and Veziroglu, 2001). Having the highest energy content in comparison to other gases, hydrogen can easily react with oxygen to produce energy and water (Reilly *et al.*, 2014). Even though hydrogen may not be readily available in a molecular form, it can be produced from numerous sources such as fossil fuels, biomass and water. At present hydrogen is produced mainly from water by electrolytic, thermochemical and biological processes (Sydney *et al.*, 2014).

Water electrolysis processes include high pressure (Laoun, 2007), high temperature (Doenitz *et al.*, 1980), alkaline water (Zeng and Zhang, 2010) and polymer electrolyte membranes (Grigoriev *et al.*, 2006) that can be used to produce hydrogen. Electrochemical hydrogen production involves splitting water molecules into oxygen and hydrogen gas using electric current. Various anode and cathode electrodes used in this process must be inexpensive, easily accessible, have non-polluting properties and exhibit good electrochemical stability (Carmo *et al.*, 2013). Accumulated hydrogen can then be integrated in fuel cells to generate energy (Momirlan and Veziroglu, 2002).

Another area of interest is thermochemical hydrogen production which is a combination of high temperatures (1500-2500°C) with chemical reactions to separate hydrogen and oxygen in water (Bohnert *et al.*, 2000). Even though these options seem to be reasonable to produce hydrogen, the amount of energy required is quite vast and its practise is not always environmentally friendly (Hatta, 1991).

Alternatively, biological hydrogen production methods are designed to operate at standard temperatures and pressure and is thus less energy demanding. They can also utilize waste material to generate hydrogen which in turn promotes waste management. Therefore, renewable energy production can be a simpler, cheaper, inexhaustible and an eco-friendly alternative (Dasa and Veziroglu, 2001).

2.2 Biological hydrogen production

Biological hydrogen production involves the use of waste material coupled with microbial activity to produce biohydrogen (Goud *et al.*, 2014). All biological hydrogen processes are dependent on hydrogenases which are the molecular base for hydrogen production because of its ability to catalyse reversible oxidation of molecular hydrogen (Seviour and Nielsen, 2010).

Hydrogenases are situated in either the cytoplasm or periplasm of the microbial cell and are involved in both consumption and generation of hydrogen. They can be further divided into Fe hydrogenases, Ni-Fe hydrogenases and Ni-Fe-Si hydrogenases (Conference and Khosrowpour, 2001). Hydrogenases are able to evolve molecular hydrogen *in vitro* if electron mediators are present or hydrogen can be oxidized to protons. The quantity and activity of these enzymes can restrict the overall hydrogen production process (Hallenbeck and Benemann, 2002, Vijayaraghavan and Mohd Soom, 2006).

Biological production methods can be classified into two categories namely photosynthetic biophotolysis by microalgae and cyanobacteria, decomposition of organic material by facultative anaerobic bacteria or photo-synthetic bacteria through dark /photofermentation.

2.2.1 Direct and indirect biophotolysis by green microalgae and cyanobacteria

Biophotolysis of water occurs directly by green microalgae and indirectly by cyanobacteria. Cyanobacteria (prokaryotic) and green microalgae (eukaryotic) contain chlorophyll a and additional pigments that assist in capturing sunlight (Carmo *et al.*, 2013, Sanjay Kumar Gupta, 2013). By the absorption of sunlight green microalgae and cyanobacteria photosynthetically produce hydrogen (Reaction one, two and three). Photosynthesis in green microalgae produce oxygen as well as electrons which are created and transferred to ferredoxin, which in turn aids in protons being converted into hydrogen by the reversible hydrogenase enzyme. This phenomenon is known as direct biophotolysis (Oey *et al.*, 2016). These photoautotrophic microorganisms have photosystems (photosystem II and photosystem I) which work directly with the enzymes, nitrogenase and hydrogenase that perform oxygenic photosynthesis (Ferreira Rosa *et al.*, 2014). Photosystem II is responsible for water splitting and oxygen evolution, whilst photosystem I is used to reduce carbon dioxide and form

hydrogen. This reaction can be demonstrated by cyanobacteria from two instances. The first being hydrogen generation catalysed by nitrogenase reducing nitrogen to ammonium and secondly hydrogen being synthesized by hydrogenase activity (Hallenbeck *et al.*, 2012).

$2H_2O \rightarrow 2H_2 + O_2$ (1)

$12H_2O + 6\ CO_2 \rightarrow C_6H_{12}O_6 + 6O_2$ (2)

$C_6H_{12}O_6 + 12H_2O \rightarrow 12H_2 + 6\ CO_2$ (Zhu *et al.*, 2010) (3)

Although the reaction seems simple, studies have revealed that the oxygen produced during the photosynthetic process, destroys hydrogenase activity (hydrogen catalyst) thus causing reduced hydrogen production (Eroglu and Melis, 2011). Other major concerns are explosive gas mixtures of hydrogen and oxygen streams being produced, slow growth rates of photosynthetic microorganisms and the large land space needed for microbial cultivation (Levin, 2004).

2.2.2 Microbiology of photofermentation

Photofermentation is a process where the microorganisms use energy from sunlight to perform anaerobic photosynthesis. A common microorganism capable of performing photo fermentation is non-sulfur purple bacteria (Patel *et al.*, 2012). Non-sulfur purple bacteria capture light energy which is conveyed to the nitrogenase enzyme to convert organic material such as molecular fatty acids into hydrogen and carbon dioxide (Reaction Four). This reaction can only take place in the absence of nitrogen. Extensive studies have focused on the ability of Rhodobacter and *Rhodopseudomonas* spp. of producing hydrogen (Bičáková and Straka, 2012). It has the potential to perform under a variety of growth conditions. The absence of photosystem II gives them the ability to overcome the possibility of hydrogen not being produced due to the lack of oxygen-evolving capabilities.

$CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2$ (Kapdan and Kargi, 2006) (4)

However, the limitations of this method includes scarce accessibility of fatty acids, substantial amounts of energy requirement, its fermented broth may cause pollution, slow

activity of nitrogenase enzyme and reverse hydrogen oxidation (Kovacs *et al.*, 2006, Khalid *et al.*, 2011).

Therefore, after considering the implications involved in using biophotolysis or photofermentation, scientist have turned their attention towards dark fermentation. Dark fermentation proves to be a much faster method of reaction when compared to photofermentation. Not only can hydrogen be produced at a quicker pace, it can also use waste material and waste streams containing carbohydrates as substrate.

2.2.3 Microbiology of Dark Fermentation

The production of biohydrogen by dark fermentation can be accomplished by a process of anaerobic digestion (Figure 1). This process occurs by microorganisms that break down decomposable material in the absence of oxygen. The digestion route in hydrogen production is divided into three stages viz., hydrolysis, acidogenesis, acetogenesis and methanogenesis (Chandra *et al.*, 2012).

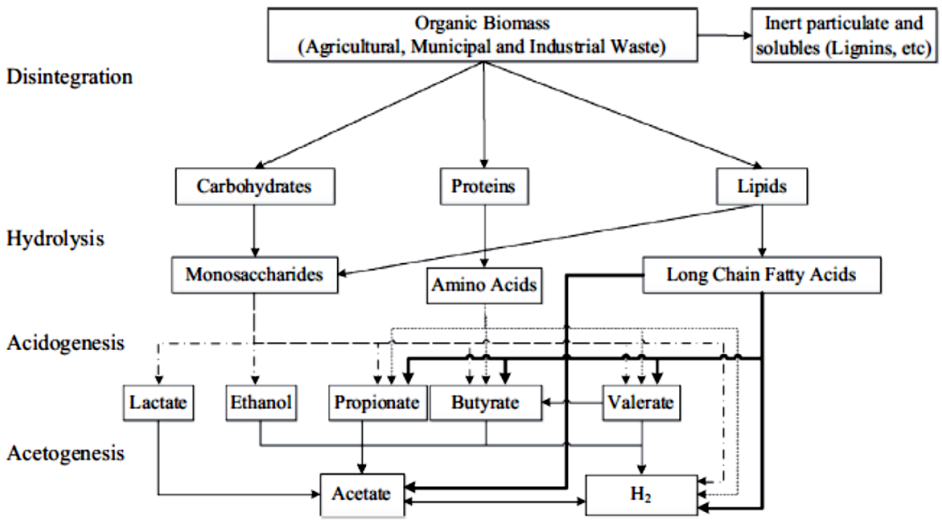


Figure 1. Biological fermentative breakdown of waste material (Ghimire *et al.*, 2015a).

2.2.3.1 Hydrolytic bacteria

The initial phase of digestion is initiated by obligate and facultative anaerobic bacteria (Ziemiński and Frąc, 2014). These hydrolytic bacteria breakdown soluble and insoluble molecules into smaller compounds which are transported into cells of fermentative microorganism for metabolism (Bitton, 2011).

2.2.3.2 Acidogenesis and acetogenesis

The formation of biohydrogen through dark fermentation is accomplished by strict/facultative anaerobic bacteria which occur in the second and third steps of fermentation. These microorganisms are responsible for the intricate biochemical process of producing end products from a particular substrate (Ahn *et al.*, 2010). The biological reactions acidogenesis and acetogenesis occur by the conversion of monomers into short-chain volatile fatty acids. Complex substrates of carbohydrates, lipids or proteins are broken down by hydrolytic enzymes (hemicellulases, cellulases, proteases, etc.) and the compounds formed are fermented into volatile fatty acids such as butyrate, acetate, propionate, lactate and ethanol (Kim and Kim, 2012).

A well-known and accepted fermentative reaction is of glucose degradation (Reaction 5) is used to explain the process in which hydrogen is produced. Glucose can be fermented into organic by-products such as acetate, formate and butyrate. Theoretically four moles of hydrogen is produced by the consumption of one mole of glucose.

$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2$ (Barca *et al.*, 2015) (5)

This theoretical yield can be reached by the fermentation of glucose to acetate and formate. In a different instance, butyrate can also be the only by-product produced instead of acetate. When butyrate is the sole by-product, only two moles of hydrogen is produced per mole of glucose. Irrespective of the type of by-product formed all reactions lead to the production of pyruvate, the pyruvate formed is produced from acetyl CoA (Azbar and Levin, 2012). The metabolism of glucose to pyruvate in almost all bacteria is carried out via the Embden-

Meyerhof-Parnas (EMP) pathway (Merlin Christy *et al.*, 2014). A crucial component of the reaction is the production of acetyl CoA from pyruvate. Acetyl CoA is the factor that determines whether the maximum yield of hydrogen is achieved or not. A further reduction of acetyl CoA to acetate or butyrate can arise by the re-oxidation of ferredoxin via the hydrogenase enzyme yielding one mole of hydrogen from one mole of ferredoxin. Additional moles of hydrogen are formed from the reduction of NADH and thus four moles of hydrogen is yielded (Ntaikou *et al.*, 2010). The key microorganisms involved in this process include heterofermentative bacteria in mixed-acid fermentations carried out by *Escherichia coli*, *Salmonella*, *Shigella* and *Proteus* species which belong to the family of Enterobacteriaceae. These microorganisms ferment glucose to carbon dioxide and hydrogen with a combination of acid end products. Other microorganism that produce hydrogen and neutral end products are *Enterobacter aerogenes*, *Aeromonas*, *Serratia*, *Erwinia*, and *Bacillus*. Clostridia (*Clostridium saccharobutyricum*, *C. thermosaccharolyticum*) and *Butyribacterium*) and *Propionibacterium* species form similar acidic by-products (Jurtshuk, 1996).

2.2.3.3 Methanogenesis

Methanogenesis is the final step of decomposition during anaerobic digestion. Methane gas is formed by microorganism known as methanogens. These organisms can be identified from the group of eukaryotes belonging to the Archaea domain (Thauer, 1998). Some common methanogenic bacteria are *Methanococcus*, *Methanobacterium*, *Methanobrevibacter*, *Methanosarcina* and *Methanospirillum* (Karakashev *et al.*, 2006). Methanogens use hydrogen as an electron donor to convert carbon dioxide to methane and acetate (Gerardi, 2003).

2.2.4 Dark-photo fermentation

This two stage fermentation process can either be sequential or combined method of biohydrogen production. The combination of dark and photo fermentation can theoretically yield 12 moles of hydrogen per mol of glucose/hexose (Redwood and Macaskie, 2006, Kosourov *et al.*, 2002). End products (volatile fatty acids and alcohols) produced during dark fermentation, can be utilized by photosynthetic bacteria as a carbon source (Lo *et al.*, 2010).

Direct conversion of these soluble metabolites can be achieved with photofermentation, resulting in reduced chemical oxygen demand (COD) content as well as an elevated yield of hydrogen. Although the theoretical yield of hydrogen seems achievable, the rate-limiting step is the slow growth of photosynthetic bacteria which can subsequently cause lower hydrogen production (Su *et al.*, 2009).

2.2.5 Factors affecting dark fermentative biohydrogen production

The fermentative hydrogen production process is influenced by many aspects such as substrate, inoculum, temperature, pH, hydraulic retention time (HRT), reactor configurations, nitrogen and iron concentration.

A. Substrate

The substrate of choice directly influences the yield of hydrogen produced as well as the overall cost of the process. Substrates of pure glucose, sucrose and starch mixtures have been well researched in dark fermentation studies. However, in renewable biohydrogen studies, sources of feedstock should be obtained from renewable resources (Ren *et al.*, 2011). Using waste as a primary source of substrate for fermentation can aid in waste treatment apart from hydrogen production. Studies (Table 1) have moved on into incorporating municipal wastewater and solid waste, agricultural residues, effluent from livestock and other waste material in to the dark fermentation process (Chen *et al.*, 2012, Jeswani *et al.*, 2012). Waste materials selected to be in biohydrogen production have to be readily available, low cost, have high carbohydrate content and be biodegradable (Kapdan and Kargi, 2006).

Municipal waste (organic fraction) contains high amounts of carbohydrates, moisture content and volatile solids. It comprises of food waste responsible for methane emissions at landfills and large quantities of sludge generated at wastewater treatment plants (Jiang *et al.*, 2013, Wang *et al.*, 2003b). Excess sludge accumulated at wastewater treatment plants can be used for bioenergy production by anaerobic dark fermentation. Utilization of such waste products is considered to be economically viable.

Table 1. Some renewable waste biomass and operating conditions used for anaerobic dark fermentation

Substrate	**Inoculum**	**Temperature (°C)**	**pH**	**Yield of H_2 (ml H_2/g sugar)**	**Reference**
Municipal solid waste	Anaerobic digestate	55	6.4	360	(Valdez-Vazquez *et al.*, 2005)
Food waste and sewage sludge	Anaerobic digester sludge	35	5.0-6.0	122.9	(Kim, 2004)
Sugar factory wastewater	Compost	60	6.0-6.5	343	(Ueno *et al.*, 1996)
Fat-rich high solid organic waste	Compost	37	7	3.3	(Lay *et al.*, 2003)
Cheese whey	Adapted anaerobic sludge	55	7	111	(Kargi *et al.*, 2012)
Rice straw	Wastewater treatment sludge	55	6.5	24.8	(Chen *et al.*, 2012)
Chicken meat	Heat treated anaerobic sludge	37	5.5	6.9	(Guo *et al.*, 2013)
Buffalo manure	2-bromoethanesulfonic acid treated anaerobic sludge	35	7.4	135.6	(Ghimire *et al.*, 2015b)

(i) Pre-treatment of lignocellulosic biomass

Hydrolysis is a chemical reaction that occurs when chemical bonds are broken down by the addition of water, which is often used to disrupt large polymers such as proteins, fats and carbohydrates into smaller components (Petrucci *et al.*, 2007). Hydrolysis is vital for frequently used substrates in biogas production such as energy crops (maize), agricultural, food and industrial wastes. Some waste materials may take a longer time to break down due to chemicals that constrain the ability of microorganisms (Ghimire *et al.*, 2015a). Agricultural wastes mainly composed of lignocellulosic materials contains large organic polymers such as carbohydrates that require degradation to be readily available for bacteria (Wettstein *et al.*, 2012). Lignocellulosic material comprise of polysaccharides that are connected by glycosidic bonds which are cleaved to yield monosaccharides through enzymatic action. Enzymes that break brown glycosidic bonds are referred to as glycoside hydrolases. Their main function is to disrupt the rigidity of the plant cell wall structures (Carrier *et al.*, 2011).

The compact plant structure (Figure 2) is made up of cellulose, hemicellulose and lignin (Agbor *et al.*, 2011). Cellulose is the main faculty of support, they are grouped together to form microfibrils that contain numerous D-glucose units in a rod-like configuration. Hemicellulose consists of a branched conformation linking arabinose, xylose (pentoses) and glucose, mannose, galactose (hexoses). They have a lower molecular weight than that of cellulose therefore can be simply hydrolysed (Barakat *et al.*, 2015). Lignin is the outer plant constituent that binds all facets of lignocellulosic material together in an irregular formation. It contains linkages of phenyl propane units that restrict enzymatic hydrolysis of plant material (Limayem and Ricke, 2012).

Research has found that a major problem in using lignocellulosic material is the inactivation of cellulase enzymes that are responsible for the division of cellulose and hemicellulose. Generally, removal of the lignin layer can increase enzyme activity during hydrolysis (Ximenes *et al.*, 2011). Therefore, in order to degrade the desirable components of the plant, an effective pre-treatment method has to be employed to release most C5 sugars that will be used as substrate during the process of biohydrogen generation. The pre-treatment procedure should be able to disrupt the lignin component of the

plant material and eliminate the possibilities of losing these valuable sugars through degradation (Hu and Ragauskas, 2012).

Agricultural waste is mainly lignocellulosic material produced when valued products of crops are harvested and the waste is cobs, bagasse, straw, peeling, etc (Mtui, 2009). Within the KwaZulu Natal region of South Africa, the sugar industry is a prominent venture that produces mass quantities of sugarcane bagasse amongst other waste products. This by-product can be an ideal source of substrate for biohydrogen generation; it contains high amounts of sugar that can easily be utilized by anaerobic bacteria. However, research has found that a major problem in using lignocellulosic material is the inactivation of cellulase enzymes that are responsible for the division of cellulose and hemicellulose.

The pre-treatment procedure should be able to disrupt the lignin component of the plant material and eliminate the possibilities of losing these valuable sugars through degradation (Figure 2) (Hu and Ragauskas, 2012).

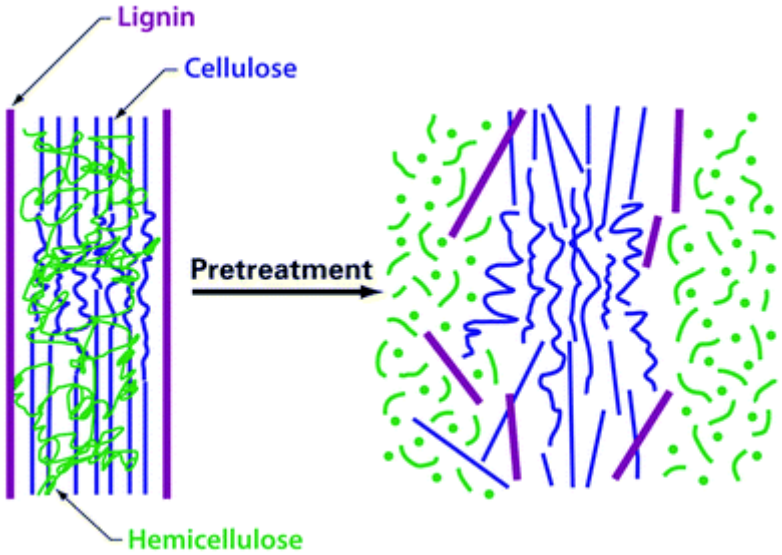


Figure 2. Schematic disruption of lignocellulosic material (adapted from (Mosier *et al.*, 2005).

Pre-treatment methods used are classified into four categories:-

Physical pre-treatment includes comminution, which is mechanical reduction by chopping grinding or milling biomass to a smaller particle size. Reduction in substrate size exposes the plant cell structure, causing an increase in surface area which favours enzymatic interaction needed for hydrolysis. Particle size can also influence mixing in bioreactors, a smaller particle size eliminates problems experienced with mixing. A suggested particle size ranges from 1-2mm, which has proven to be a considerable size for hydrolysis (Sarkar *et al.*, 2012).

Biological pre-treatment involves bioengineered microbes or employing fungi that have the ability of generating enzymes that can destroy lignin but may also remove organic substrates in the same process (Deswal *et al.*, 2014).

Chemical pre-treatment has been explored using different chemicals, mostly acids (sulphuric, hydrochloric, sulfamic acid. etc.), alkali (sodium hydroxide, calcium hydroxide and potassium hydroxide) and ionic solutions. Acid treatment works to break down hemicellulose and the link found between hemicellulose and lignin. Unlike acid pre-treatment, alkali pre-treatment causes swelling of celluloses and a fraction of lignin to solubilise. Thus having considerable effects on the structure of lignocellulosic material (Lin *et al.*, 2014, García *et al.*, 2014).

Physicochemical pre-treatment is a combination of mechanical, chemical and thermal processes such as ammonia fibre explosion, steam explosion or liquid hot water with chemical treatment (Brodeur *et al.*, 2011). Steam and pressure plays an important role causing cells to rupture at temperatures ranging from 160-220°C for 5-60 minutes (Waldron, 2014). When the efficiency of all treatments is compared, physicochemical pre-treatment is the most favoured due to its effectiveness.

B. Hydrogen producing microorganisms

Hydrogen producing inoculum is a critical factor for start-up of the hydrogen production process. Microorganisms that are capable of producing hydrogen, occur in environments such as soil, sludge or compost, which is a prospective source of inoculum for fermentative hydrogen production (Sinha and Pandey, 2011).

Microorganisms that carry out dark fermentation have metabolic pathways that are strictly or facultative anaerobic (Piemonte *et al.*, 2014). Hydrogen can be produced through pure and mixed populations of microorganisms from fermentation of several substrates. Most studies have reported *Clostridium* and *Enterobacter* species being used as inoculum for dark fermentation studies performed with pure cultures (Rittmann and Herwig, 2012). *Clostridium* species are gram positive strict anaerobes, rod shaped and endospore formers. Whereas *Enterobacter* species are rod shaped gram negative rods and exist as facultative anaerobes (Wang and Wan, 2009). The major disadvantage of using pure cultures is the strict handling and control of cultures under aseptic environments which can be time and energy consuming (Kalia, 2016).

Use of mixed cultures of microorganisms is considered as a more practical option of hydrogen production as they are easier to operate and control (Ghimire *et al.*, 2015a). Mixed bacterial cultures from wastewater or waste treatment processes are preferably used for biohydrogen production. However, under anaerobic conditions using such a system can also favour the growth of hydrogen consuming bacteria such as methanogenic and homoacetogenic microorganisms (Sträuber *et al.*, 2012). Thus, in order to eliminate this occurrence, sludge should undergo pre-treatment to suppress or eliminate bacterial activity of hydrogen consuming bacteria while still conserving hydrogen producing communities (Wang and Wan, 2008a).

A beneficial characteristic of certain hydrogen producing microorganisms is its ability to form spores under harsh conditions (Venkata Mohan *et al.*, 2008). Most of the known hydrogen consuming bacteria are non-spore forming bacteria, hence selection of appropriate pre-treatment methods would aid in selection of spore forming hydrogen producing bacteria from the mixed inoculum.

(a) Sludge Pre-treatment methods

Anaerobic sludge pre-treatment methods include single or combination of methods such as chemical, thermal and alteration to process control. These treatment methods have a distinctive mode of action as discussed below.

(i) Methanogenic chemical inhibitors

Chemicals such as 2- Bromoethanesulfanoate (BES) and chloroform are the two common inhibitors used to prevent methanogenic activity (Liu *et al.*, 2011). BES being an analog of coenzyme M in methanogens making it methanogenic specific (Chen *et al.*, 2007). The concentration of BES depends on the reaction system used to achieve effective inhibition. Some studies have shown that a concentration of 100m*M* was sufficient in a rice root system (Conrad and Klose, 1999), whilst 50m*M* was sufficient in an thermophilic digester and a higher concentration was required for hydrogentrophic methanogens (Zinder *et al.*, 1984). Treating sludge at concentrations of up to 100m*M* however would be too expensive during large-scale production. Few other studies have found that using chloroform can block the production of methane and carbon dioxide by disabling the function of corrinoid enzymes.

However, studies have revealed that chloroform can also inhibit activity of other groups of microorganisms, thus disrupting the acetyl-CoA pathway which is also responsible for hydrogen production (Scholten *et al.*, 2000). Although the mechanisms of chemical inhibitors have been investigated in detail, their application still needs to be investigated such as their appropriate concentration levels, properties and adverse effects.

(ii) Acid and alkaline treatment

Acid and alkaline chemical pre-treatment methods depend on the action of the chemical structure of the solvent. Acid treatment is influenced by the free H^+ whilst the alkaline treatment depends on the free OH^- in solution. This method hydrolyses the cell components and increases the organic matter within the cells (Appels *et al.*, 2008). Many cases have used such treatment for sludge from wastewater treatment plants. Since conventional wastewater treatment processes maintain pH at 7, at this pH methane producers proliferate therefore to reduce the methane production rate, pH must be decreased to below 6.3 or increased above 7.8. Thus, changing the pH of anaerobic sludge inhibits the activity of hydrogen consumers whilst hydrogen producers are found to have increased hydrogen production within 24 hours. However, these methods require stringent pH control and would take a longer period of time to establish the hydrogen producing consortium (Chang *et al.*, 2002, Chen *et al.*, 2002).

(iii) Thermal treatment

Heating sludge has been the most used method to screen for hydrogen producing bacteria. This method is dependent on high temperatures by thermally suppressing methanogenic *Archaea* (non-spore forming bacteria) and enriching sporulating hydrogen producing bacteria (*Clostridia*) (Venkata Mohan, 2009, Zhu and Béland, 2006). Cell components of microorganisms get disrupted with heat causing solubilisation of cell constituents. Various heating temperatures and time periods have been studied ranging from 75°C to 121°C (Chang *et al.*, 2002, Wang *et al.*, 2003a) for duration of 15 minutes to two hours (Lay *et al.*, 1999, Fan *et al.*, 2004). Heat treatment kills vegetative cells of non-spore forming microorganisms which could also eliminate hydrogen producing microorganisms that lack the ability of sporulating. However, heat treatment can also activate dormant spores of hydrogen producers and once optimal conditions are reached these cells can germinate to produce hydrogen (Watanabe *et al.*, 1997). Some cases have shown heat treated microorganisms displaying reduced hydrogen yields and substrate conversion which may have been due to the reduction of hydrogen producing bacteria (Zhu and Béland, 2006, Kraemer and Bagley, 2007).

C. pH and Temperature

Factors such as pH and temperature affect the metabolic pathways for hydrogen synthesis and also simultaneously inhibiting the occurrence of methanogenic microorganisms within the reactor (Khanal *et al.*, 2004, Hu *et al.*, 2005). At an acidic pH below 6, inhibition of methanogenic activity occurs under mesophilic and thermophilic conditions. However, under thermophilic condition inhibition of homoacetogenic activity will be achieved at pH 5.5 (Luo *et al.*, 2011). Therefore controlling pH and temperature is important to reach high biohydrogen conversion rates by eliminating hydrogen consumers.

pH in particular can influence the metabolic pathway affecting the hydrogenase activity. An acidic pH can also affect the hydrogenase enzyme even though it is used for methanogenic inhibition. An optimal pH range for biohydrogen production ranges from pH 4 to 9 (Khanal *et al.*, 2004, Lee *et al.*, 2002b). Operational pH can also define the metabolic by-products produced along with hydrogen. Reported studies have stated that acetate and butyrate are most common end products of fermentation (Han and Shin, 2004b). A neutral operational pH favoured the acetate pathway (Han and Shin, 2004a, Valdez-Vazquez *et al.*, 2005) whilst under acidic pH studies favoured butyrate production (Luo *et al.*, 2010b, Tang *et al.*, 2008).

Similarly, temperature is another most influential factor that could affect hydrogen production. Studies have shown that temperature can also affect metabolic pathways thus shifting end products of fermentation. Mesophilic (35°C) (Fang *et al.*, 2002a), thermophilic (55°C) (Ratti *et al.*, 2015) and extreme thermophilic (>65) (Kongjan and Angelidaki, 2010) operational temperatures have been studied to determine the effect on biohydrogen generation. A higher hydrogen yield was reported during thermophilic fermentation than when compared to mesophilic conditions (Liu *et al.*, 2008a). Production of fermentative by-products differed by acetic acid being prominent during thermophilic fermentation and butyrate being formed during mesophilic digestion. Extreme thermophilic experiments showed a similar result of acetic acid dominance at pH 7 (Liu *et al.*, 2008b).

D. Hydraulic Retention time

Hydraulic Retention time directly influences substrate hydrolysis and the production of intermediate products thus affecting the hydrogen generation process. HRT can also be used to control methanogenic activity (Kim *et al.*, 2006). Fermentation time is a key factor to restrict methanogenesis during acidogenic hydrogen production. Various studies have used a short HRT at an acidophilic pH (O-Thong *et al.*, 2011). An optimum HRT was found between eight to fourteen hours yielding increased hydrogen production with methanogenesis inhibition. The difference in growth rates of hydrogen consuming and producing microorganisms, makes it possible to use HRT as a parameter to resist methane producing microorganisms (Ueno *et al.*, 1996, Hussy *et al.*, 2003, Liu and Fang, 2003, Chen and Lin, 2003). Hydrogen production increases at a low HRT due to wash out of methanogens (Liu *et al.*, 2008a). However, the optimum HRT for biohydrogen production is still dependent on the type of substrate and its biodegradability.

E. Iron concentration

During fermentative biohydrogen production, bacteria require vital micronutrients for hydrogen metabolism. Some of these essential nutrients include sodium, zinc, magnesium and iron (Sinha and Pandey, 2011). Iron plays an important role in hydrogenase formation by electron transport which produces more hydrogen. In the presence of iron, acidogenic metabolism is directed by the iron sulphur protein in ferredoxin which acts as an electron carrier to oxidize pyruvate to acetyl CoA and carbon dioxide and as a proton reducer to molecular hydrogen. However, in iron deficient conditions flavodoxin replaces ferredoxin as an electron transporter thus reducing the possibility of hydrogen production. Therefore, it is essential to supplement iron into fermentative hydrogen production to improve ferredoxin activity (Lee *et al.*, 2002a). One of the studies conducted demonstrating the supplementation of 25 mg/L of iron resulted in enhanced hydrogen yield with 391 to 408 ml/g of glucose utilized. In addition, it was reported that an increased iron concentration could also negatively affect hydrogen production (Dabrock *et al.*, 1992).

F. Reactor configurations

Reactor configuration is one of the most important factors that affect the environment of microorganisms within the reactor (Ntaikou *et al.*, 2010). Many studies have been conducted using three modes of operation which are batch, semi-continuous and continuous reactors for simple operation and effective control of biohydrogen production. However, at large scale production, a continuous production process would be required. Researchers have developed several bioreactor configurations based on operational conditions such as substrate, culture temperature (mesophilic or thermophilic) and feeding mode (single or multiple feeds) (Motte *et al.*, 2014). A number of biohydrogen bioreactor types have been explored in the past such as Continuous Stirred Tank Reactor (CSTR), Membrane Bioreactor (MBR), Anaerobic Baffled Reactor (ABR), Fluidized-bed Bioreactor and Upflow Anaerobic Sludge Blanket reactor (UASBR).

(i) Continuous stirred tank reactor

The continuous stirred tank reactor is the most widely used design for continuous biohydrogen production from several substrates (Yuan *et al.*, 2009, Fang *et al.*, 2002c, Seengenyoung *et al.*, 2014, Reungsang *et al.*, 2013). The reactor design compensates for complete mixing between the substrate and biomass thus allowing for even pH and temperature control. On the other hand, the process configuration of the reactor can strongly influence the fermentation process (Li and Yu, 2011). For instance, CSTR is unable to maintain significant levels of fermentative biomass. It was shown that using attached sludge CSTR was more stable than suspended sludge CSTR because the attached sludge is able to retain a high biomass concentration (Ren *et al.*, 2009). Another limitation is biomass washout that occurs during short hydraulic retention times, consequently restricting hydrogen production rates. However, researchers have demonstrated that hydrogen producing sludge can be agglutinate into granules in a well-mixed CSTR or using silicone immobilized sludge to treat synthetic wastewater containing sucrose. The reactors were operated at various conditions of HRT (from half an hour to six hours), pH (5.5-6.6) and temperature at (26-40°C) (Fang *et al.*, 2002b, Wu *et al.*, 2006) to enhance biomass retention and hydrogen production rate.

(ii) Membrane Bioreactor

The reactor can be classified into two types viz., external cross-flow type and submerge type (Jung *et al.,* 2011a). The MBR is advantageous because of high biomass accumulation that uses a greater amount of organic substrate, a smaller reactor volume and reduced loss of microorganisms in the effluent (Oh *et al.,* 2004). The submerged type reactor has been used more often due to it being less energy intensive and requires less membrane maintenance (Lee *et al.,* 2009a). Hydraulic and sludge retention time (SRT) are two main factors to consider for biohydrogen production in a membrane bioreactor (Bakonyi *et al.,* 2014). Previous studies had demonstrated that an increased efficiency of biohydrogen was maintained at a longer SRT along with a shorter HRT (Hafez *et al.,* 2009). This is due to a greater population of hydrogen producing microorganism which would result in higher gas production and substrate utilization (Melin *et al.,* 2006). Stable hydrogen production was also reported in submerged reactor using glucose at an HRT of 9 hours. However, this study had also proven that a longer SRT can result in accumulated extracellular polymeric substances (EPS) which can inhibit hydrogen evolution within the reactor (Lee *et al.,* 2010). Whilst another investigation using 450 days of SRT had shown no loss in hydrogen generation which can imply that the optimal SRT and HRT are system specific (Lee *et al.,* 2008).

(iii) Fluidized-bed Bioreactor

The Fluidized-bed bioreactor was mostly used for wastewater treatment owing to its low HRT and high efficiency with high biomass concentration (Hickey and Owens, 1981). Later on investigations were conducted using fluidized-bed bioreactors for hydrogen production by immobilizing hydrogen producing bacteria in granular activated sludge and expanded clay (Lin *et al.,* 2006, Wu *et al.,* 2003). In a study conducted by Zhang et al., 2007, granular activated sludge and biofilm based biomass was used to produced hydrogen from glucose. The results obtained indicated that biofilm based studies produced a higher hydrogen production rate than that of granular sludge (Zhang and Shen, 2007b). In other cases, expanded clay was more favorable as a support medium yielding a hydrogen production rate of 1.28 L H_2/L/h at a HRT of one hour (Shida *et al.,* 2009, Barros *et al.,* 2010).

(iv) Upflow Anaerobic Sludge Blanket Reactor

The development of the UASBr reactor was first developed for biogas production by treating various streams of wastewaters (Wang *et al.*, 2007b). The reactor has a longitudinal structure with gas, liquid and solid separators. The design was first used in the year 2002, showing stable performance of hydrogen production (Fang *et al.*, 2002b). Other studies were conducted thereafter using synthetic wastewater as a substrate (Chang and Lin, 2004). The highest hydrogen yield was found at 0.75 mol H_2/mol hexose at a HRT of eight hours (Mu *et al.*, 2006a, Mu *et al.*, 2006b). Research was also conducted using actual wastewater in UASB reactor (Huang *et al.*, 2004, Jung *et al.*, 2011b, Yu *et al.*, 2002).

G. Enhancing bio-hydrogen production with nanoparticles

Nanoparticles have recently received much consideration to enhance the bioactivity of hydrogen producing bacteria (Han *et al.*, 2011a). Nanoparticles can be synthesized by sputtering to chemical vapour deposition and most popular microemulsion technique. The microemulsion technique is a useful wet chemical method that is dependent on the nature of the surfactant to participate in self-assembly into a micelle. Synthesizing such structures are advantageous because of their protective and magnetic properties (Carpenter, 2001). Nanoparticles such as gold, iron, metal and oxide have shown to improve kinetics of the bioprocess through its ability to react with electron donors and increase the activity of microorganisms under anaerobic environments (Xu *et al.*, 2012, Beckers *et al.*, 2013a, Zhang and Shen, 2007b).

Metals and metal oxide nanoparticles have been of interest because of low cost, unique physical and chemical properties. Iron nanoparticles have shown much potential for hydrogen production as iron is easily available as it is a common metal. Since iron has a low standard redox potential it can be readily oxidized by water. According to Reaction 6, iron reacts once in contact with water to produce hydrogen gas under ambient conditions, one mole of hydrogen is generated when one mole of iron is consumed (Reardon, 1995).

$$Fe^0 + 2H_2O \rightarrow Fe^{2+} + 2OH^- + H_2 \qquad (6)$$

Iron oxide nanoparticles can be represented in different forms such as magnetite (Fe_3O_4), hematite (α-Fe_2O_3) and maghemite (γ-Fe_2O_3) (Teja and Koh, 2009). Researchers have proposed that an interaction between iron oxide nanoparticles and hydrogen producing bacteria exists, thus resulting in rapid hydrogen production rate (Han *et al.*, 2011a). The claim states that nanoparticles would behave has "electron sinks" that would further reduce protons to hydrogen (Mu and Chen, 2011). Investigations using hematite nanoparticles found that an optimum concentration of 200mg/L yielded 3.21 mol H_2/mol sucrose (Han *et al.*, 2011a). Maghemite nanoparticles have also being explored by using actual starch wastewater to enhance hydrogen production rate in a mixed culture system. Results showed that cultures immobilized with nanoparticles had an increased yield of hydrogen than that of unaltered cells (Nasr *et al.*, 2015).

H. Techniques used to detect microorganisms from anaerobic sludge

Culture dependent techniques have been used to identify key hydrogen producing microorganisms by traditional methods. These techniques are based on morphology, nutritional requirements, pathogenic properties, etc. (Elsharnouby *et al.*, 2013). However, using traditional methods have limitations, as it is difficult to work with isolated pure cultures. Not all hydrogen producing microbes are culturable thus limitations lie in assessing the real time microbial activity using conventional methods.

Molecular techniques present have advantages over conventional techniques and is realiable (Sanz and Köchling, 2007). Fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), polymerase chain reaction and quantitative polymerase chain reaction (QPCR) are some of the recent techniques that are being used for detection and quantification of microbes from environmental samples.

(i) Fluorescent in situ hybridization

Fluorescent in situ hybridization is an excellent tool to study microbial populations by microscopic technology using short sequences of 16S rRNA-targeted oligonucleotide probes labelled with a fluorescent dye (Olsen *et al.*, 1986, Amann *et al.*, 1995). Cultures collected

from almost all environments can be identified by morphology and quantified according their taxonomic level to confirm presence of microorganisms (Karło and Ziembińska, 2013). Fluorescent labelled probes can be used to observe anaerobic microorganisms at a species level or by specific groups (Fernadez *et al.*, 2008). Studies have used FISH to monitor several microbial communities of hydrogen producing bacteria. During the fermentation of starch, mesophilic and thermophilic sludge samples were analysed by FISH to target most species of *Clostridium histolyticum,* group and the domain Eubacteria (Zhang *et al.*, 2003). Hydrogen producers were also identified in a study using palm oil effluent for hydrogen production. The predominant microorganisms hybridized were *Thermoanaerobacterium* spp. and gram-positive bacteria belonging to the phylum of Firmicutes (Nitipan *et al.*, 2014). FISH has been a useful means of observing methanogenic bacteria before and after sludge pre-treatment. In an investigation using acid pre-treatment, FISH demonstrated the effectiveness of the treatment by quantifying the ratio of *Clostridium* species to that of Archaea (hydrogen consuming bacteria) (Lee *et al.*, 2009b).

(ii) Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis is most commonly used to monitor the microbial community shifts within the system by a molecular finger printing method (Muyzer *et al.*, 1993). This is based on mobility of DNA fragments on agarose gel according to their size which form band patterns that represent the diversity of the sample. Sequencing and phylogenetic analysis of the bands can be used to interpret the composition of the microbial community (Muyzer and Smalla, 1998). Several studies have used DGGE to monitor hydrogen producing bacterial populations. An investigation using sludge compost detected 86 microorganisms which were further divided into 9 distinct groups by genetic fingerprinting. The predominant isolates belonged to the cluster of thermophilic *Clostridium* and *Bacillus* (Ueno *et al.*, 2001). A study conducted with mesophilic hydrogen producing sludge identified 96 clones screened by DGGE and thereafter sequenced for phylogenetic analysis. Most of the clones were associated with three species of *Clostridium,* Enterobacteriaceae and *Streptococcus bovis* (Fang *et al.*, 2002a). Even though this method of monitoring is favoured, its limitation lies in the long periods of time taken to process multiple clones of species which cannot be quantified (Sanz and Köchling, 2007).

(iii) Quantitative polymerase chain reaction

Quantitative polymerase chain reaction or real-time polymerase chain reaction is a simple method used to quantify target microorganisms during biological processes. The technique allows for quantification of functional gene markers within a microbial community. Quantification ranges from the domain level down to an individual species (Smith and Osborn, 2009). This technique displays the concentration of amplified products after each PCR cycle by using a fluorescent dye to reflect the concentration of the product in real-time assay (Zhang and Fang, 2006). This quantitative data produced can indicate variation in gene abundance and expression which can be used to compare variations in biological activity during fermentation. Thus the expression of the hydrogenase gene present in hydrogen producing bacteria was analysed using QPCR. Gene expression of hydrogenase was monitored during fermentation of treated and untreated activated sludge, resulting in higher expression of genes found in treated sludge after twenty four hours of fermentation (De Sá *et al.*, 2011).

Quantitative polymerase chain reaction was also useful to study the survival rate of hydrogen producing bacteria amongst other bacteria under mesophilic conditions. Hydrogen producing *E. cloacae* maintained a stable cell number which influenced the microbial structure of the system (Acs *et al.*, 2015). Hydrogen producing microbial populations in batch reactions can also be detected by QPCR and used to estimate the time taken for hydrogen producing cells to generate and the rate that hydrogen is produced (Fang *et al.*, 2006). Quantitative polymerase chain reaction is a robust, reproducible and a highly sensitive method to track microbial changes under different experimental conditions (Smith and Osborn, 2009).

CHAPTER THREE:
SCREENING AND OPTIMIZATION OF A SUITABLE PRE-TREATMENT METHOD TO HYDROLYSE SUGARCANE BAGASSE

3.1 Introduction

One of the major challenges of using sugarcane bagasse as a substrate for dark fermentation is that the available sugars are locked in the complex cellulose and hemicellulose components within the plant fibre structure (Manzoor *et al.*, 2012). Thus, pre-treatment is needed to disrupt the recalcitrant structure of this lignocellulosic material, by breaking the lignin seal and decreasing crystallinity of cellulose. Various pre-treatment methods have been employed previously, which includes physical (mechanical and extrusion), chemical (alkali, acid, ionic liquid, etc.) or physical–chemical (steam explosion, ammonia fiber explosion, microwave, etc). The chemical cooking process is the most frequently used method to separate lignocellulosic components (Fernandez, 1996). It aids in enlarging the inner surface area of the substrate by solubilization or hemicellulose and degradation of lignin (Candido *et al.*, 2012).

Previous research on bagasse concentrated on using acids and alkalis to hydrolyse sugarcane bagasse material, however, the amount of sugar extracted is limited by the strength of acid or base used, reaction time and temperature (Chen *et al.*, 2013). Acids (adding H_2SO_4 or HCl) can be used as catalysts for breaking down heterocyclic ether bonds between sugar monomers in the polymeric chains formed by cellulose and hemicellulose. Alkaline pre-treatment (using NaOH or $Ca(OH)_2$) can remove lignin from biomass, thus improving the reactivity of the residual polysaccharides and eliminating acetyl groups and various uronic acid substitutions on hemicellulose (Zeng *et al.*, 2014). In addition, using steam explosion (or hydrothermal) method, biomass is treated with high-pressure saturated steam, after which the pressure is swiftly reduced, which makes the materials undergo an explosive decompression (Agbor *et al.*, 2011). Usually, temperatures between 160 and 240°C and pressure between 0.7 and 4.8 MPa are employed (Fangkum and Reungsang, 2011).

Microwave is an alternative heating method used since it has easy operation and high heating capacity (Binod *et al.*, 2012). Generally, microwave pre-treatment is carried out at

temperatures greater than 160°C (Kitchaiya *et al.*, 2003). Studies have reported that microwave treatment can change the structure of lignocellulosic material and increase the possibilities of enzymatic activity (Xiong *et al.*, 2000).

To carry out these processes, much thought must be put into making the methods cost-effective and less energy intensive. Thus, the objective of this chapter was to screen and optimize pre-treatment methods for maximizing the release of fermentable sugars from bagasse. Pre-treatment such as weak acid/alkali at different time intervals in steam and microwave treatments were evaluated. In this study, statistical experimental design techniques (main effects plot, interaction plot and multi-vari chart) as well as response surface methodology was used to determine the effect of concentration and reaction time.

3.2 Materials and methods

3.2.1 Feed stock

Fresh sugarcane bagasse used in this study was received from a local vendor, Durban, South Africa. Samples were sun-dried, blended, and milled to a size less than 2 mm. The sieved samples were stored at room temperature for further analysis. The compositional analysis of native sugarcane bagasse was determined following the method of National renewable energy laboratory below.

3.2.2 Determination of moisture content

The moisture content was determined gravimetrically. Two grams of bagasse was placed into previously dried porcelain crucibles and dried at 105°C for 4 hours. The weight of the samples was measured at room temperature (Chong *et al.*, 2013).

3.2.3 Determination of ash content

The ash content was determined gravimetrically. Porcelain crucibles were placed into a furnace at 575°C for 4 hours and then removed and left to cool for an hour in a desiccator. Crucibles were then weighed before adding 2g of sugarcane bagasse (previously dried at 105°C). The crucibles were placed on a clay triangle and stand over a flame. As soon as smoke appeared the smoke was ignited and the sample was left for more smoke to appear. The crucible was then allowed to cool before placing it back into the furnace at 575°C for 24 hours. The crucible were removed the next day and placed in a desiccator for an hour to cool then weighed (Assawamongkholsiri *et al.*, 2013).

3.2.4 Steam pre-treatment

The lignocellulosic materials were steam pre-treated with acid and base hydrolysis. Both treatments were performed using a autoclave at 121°C and 1.4 kg/cm^3. The steam based acid hydrolysis was conducted in 250 mL Erlenmeyer flasks using sulphuric acid (1st experiment) and hydrochloric acid (2nd experiment) at low concentrations 0%, 0.25%, 0.5%, 0.75% and 1%. These reactions were performed at three different time intervals 30, 60 and 90 min. However, for the base hydrolysis, sodium hydroxide (3rd experiment) and calcium hydroxide (4th experiment) were used in the concentrations of 1%, 2% and 3% at longer time intervals of 60, 120 and 180 min.

3.2.5 Microwave pre-treatment

The Microwave hydrolysis (acid treatment and alkali treatment) was carried out in Teflon tubes (90 mL) using a microwave oven (Milestone, Start D). Microwave-acid pre-treatment was carried out using 0%, 0.25%; 0.5%; 0.75% and 1% sulphuric acid (5^{th} experiment) and hydrochloric acid (6^{th} experiment). The tubes were kept in a microwave at power 100 W for different residence time 15, 30 and 45 min. For microwave-alkali treatment, 1%, 2% and 3% sodium hydroxide (7^{th} experiment) and calcium hydroxide (8^{th} experiment) were used at the power 180 W for different reaction (60, 120 and 180 min) periods.

3.2.6 Statistical analysis

Design of experiments was selected to determine the individual and interactive effects of time and acid/alkali concentration dilution that could affect the sugar yield (SY). The functions undertaken in the current study were interaction, multi-vari and main effect (www.mathworks.com/help/stats/). An interaction plot displays the levels of one variable on the X axis and has a separate line for the means of each level of the other variable. The Y axis is the dependent variable. A main effects plot was selected to examine differences between level means for concentration% and reaction time. A main effects plot graphs the response mean for each factor level connected by a line http://www.minitab.com. Multi-vari chart was used to present analysis of variance data in a graphical form.

The response surface method was implemented to estimate the glucose yield over independent variables time (X_1) and dilution percentage (X_2). The quadratic model for predicting the optimal point is expressed in Equation 1. The conditions used for predicting maximum or minimum SY was calculated from function *fminsearch* in MATLAB (R2009a).

$$Y = A + B \times X_1 + C \times X_2 + D \times X_1 X_2 + E \times (X_1)^2 + F \times (X_2)^2 \qquad (1)$$

Where Y: response variable; A: interception coefficient, B and C: linear terms; D: interaction coefficient; E and F: quadratic terms; and X_1 and X_2 represent the variables studied.

3.2.7 Analytical methods

All the pre-treatment experiments were performed in triplicate, having a mass ratio of 1:15 of solid (g of dried sugarcane bagasse) to liquid (mL of acid or base solution). The hydrolyslate was filtered with Whatman (No. 1) filter paper, and the filtrate was neutralised (pH 7.0±1.0) for further analysis. Sugar contents were determined using HPLC (Merck Hitachi Lachrom) equipped with a refractive index detector and Aminex HPX-87H column (300 x 7.8 mm) at 40 °C. A 5 mM sulphuric acid was the eluent of choice which was measured at a flow rate of 0.60 mL/min.

3.2.8 SEM analysis

Samples of untreated sugarcane bagasse and treated bagasse was analysed by SEM to record the morphology characteristics of the bagasse before and after treatment. Prior to analysis, samples were dried for an hour in an oven at 50°C and thereafter placed onto carbon ribbon which was held down by a sample holder. The surfaces of the samples were made electrically conductive by adding a gold palladium coating at a sputter current of 3mA for 60 sec, using a Quorum (Q 150R ES) and images were observed by a scanning electron microscope (Zeiss Ultra Plus FEG SEM) at a voltage of 5.00 kV.

3.3 Results and discussion

Percentages of total solids, moisture and ash were 91.82±0.35 %, 8.18±0.35 % and 1.91±0.07 %, respectively. The high cellulose content (>45%) indicates the high potential of the sugarcane bagasse for its conversion. The chemical analyses indicate that the main components in the raw bagasse used in this study are in good agreement with those mentioned in previous studies (Barros Rda *et al.*, 2013, Binod *et al.*, 2012, Jackson De Moraes Rocha *et al.*, 2011).

3.3.1 Steam-acid treatment using sulphuric acid (1st experiment)

As shown in Figure 3, the mean sugar yield (MSY) from steam-acid hydrolysis tests was higher compared to the control, which showed a value of 131.7 mg/g. When the H_2SO_4 concentration was increased to 0.25%, the MSY significantly increased to 193.9 mg/g which further peaked to a MSY of 224.6 mg/g at 0.5% H_2SO_4. However, further increase of concentration to 1% H_2SO_4, MSY retarded again to 199.0 mg/g.

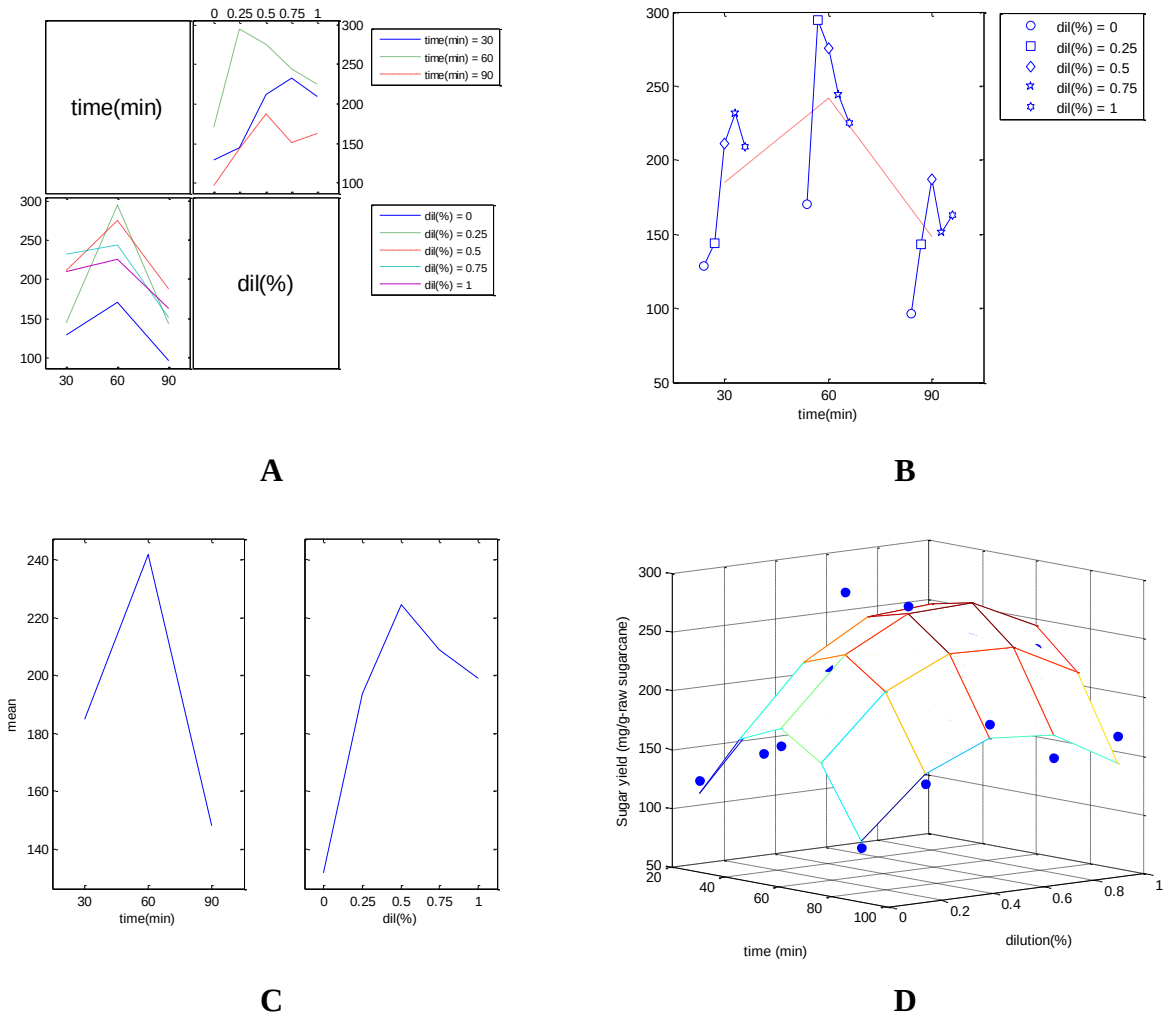


Figure 3. Interaction of hydrolysis time and sulphuric acid concentration on the sugar yield from bagasse. (A) Interaction plot, (B) Multi-vari chart, (C) Main effects plot and (D) 3D surface plot.

These results indicate that the hydrolysis condition of 0.5% H_2SO_4 was suitable to extract the sugars contained in the lignocellulosic biomass. Moreover, the MSY elevated from 184.9 mg/g to 241.9 mg/g with increasing hydrolysis time from 30 min to 60 min, respectively, indicating the potential positive impact of contact time on steam-acid hydrolysis process. However, a sharp decline in the MSY (i.e. 148.2 mg/g) owing to a further increasing time to 90 min, implies the negative effect of a relatively high hydrolysis time. The results of steam-acid treatment showed that the optimum treatment time and concentration percentage was 60 min and 0.25% H_2SO_4, whereas the least was at 90 min and 0.25% H_2SO_4. At the optimal condition, sugar content showed glucose (0.70 g/L) and xylose (1.85 g/L).

3.3.2 Steam-acid treatment using hydrochloric acid (2nd experiment)

Using 0.25% HCl, the highest MSY of about 275.20 mg/g was achieved at 60 min (Figure 4). This result is equivalent to a 40.8% relative enhancement compared to MSY observed at reaction time 30 min. It is noted that a further increase in residence time negatively affected the hydrolysis process, where MSY recorded lower value of 144.0 mg/g at 90 min. The effect of concentration showed a dramatic increase of MSY from 131.7 mg/g (control) to 224.6 mg/g at a range of 0 to 0.25% HCl. After that, MSY dropped linearly to 144.1 mg/g with an additional increase to 1% HCl. This indicates that, an increase in concentration above 0.25% HCl would negatively impact the hydrolysis process.

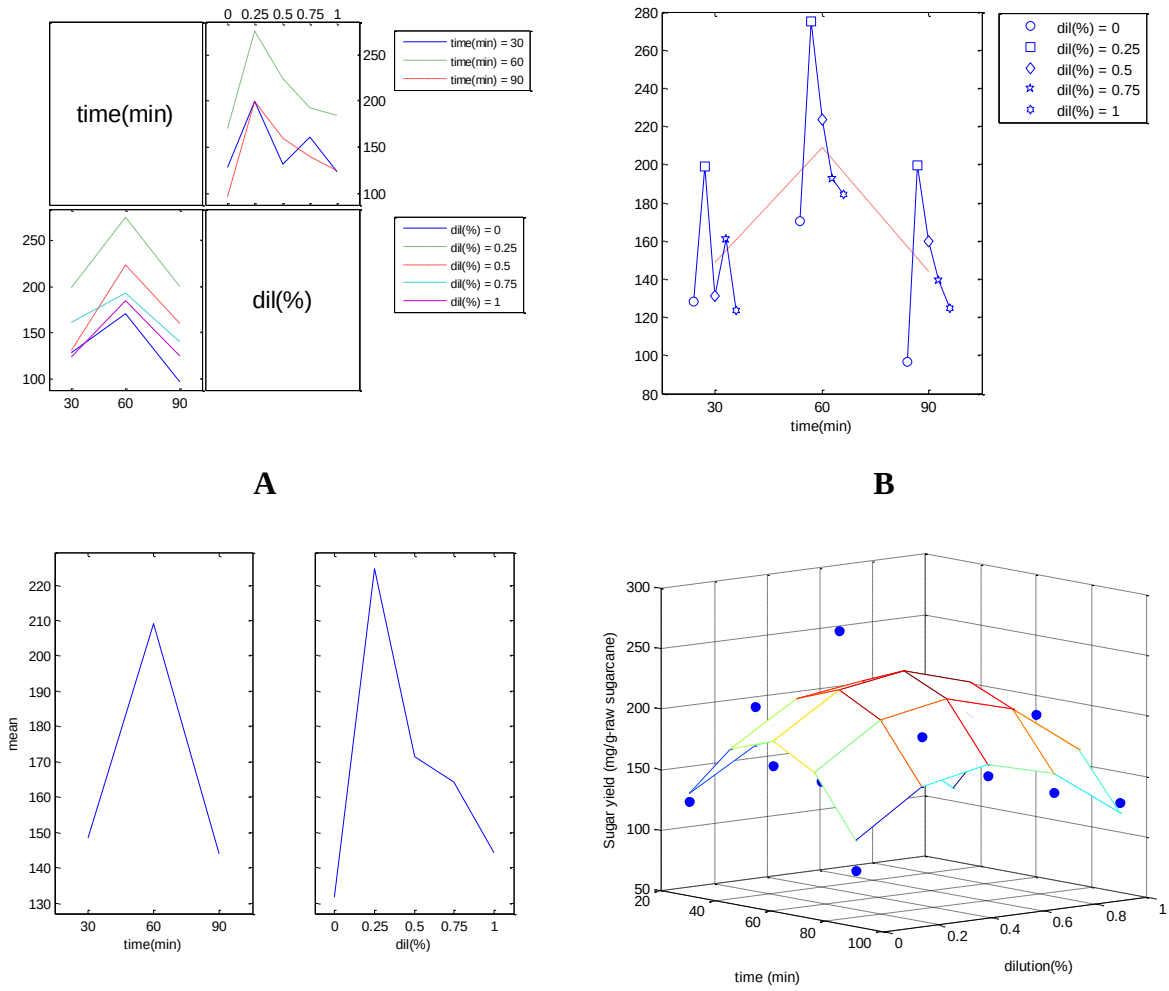


Figure 4. Interaction of hydrolysis time and hydrochloric acid concentration on the sugar yield from bagasse. (A) Interaction plot, (B) Multi-vari chart, (C) Main effects plot and (D) 3D surface plot.

Previous results were reported by (Gupta et al., 2009), where they have shown an increase in sugar release with an increase in acid concentration to 3.0%v/v. This study also stated that any further increase in acid concentration caused the increase in release of some toxic compounds or inhibitors, resulting in a decrease of sugar concentration. Our results indicated that the maximum reduced sugar yield of 275.2 mg/g was obtained with 0.25% HCl for 60 min. On the contrary, the lowest value of 123.3 mg/g was observed during 30 min steam treatment with 1% HCl. Concentrations of glucose and xylose were detected to be 0.94 g/L and 1.98 g/L, respectively after 90 min in the solution of 0.25% HCl (Appendix one, Table 14). It is evident that the optimum conditions for steam-acid hydrolysis using either H2SO4 or HCl were similar (i.e. concentration of 0.25% for 60 min steam). As listed in Appendix one, Table 10, the correlation coefficient (R^2), between measured data and simulated results

from surface response methodology, of steam-acid using H2SO4 and HCl were 0.84 and 0.68, respectively. By applying function fminsearch in MATLAB to the response surface equation, the optimum conditions for steam-acid hydrolysis were 0.64% H2SO4 for 55.7 min and 0.46% HCl for 59.4 min.

3.3.3 Steam-alkali hydrolysis using sodium hydroxide (3rd experiment)

When comparing the MSY during NaOH treatment times of 60 min with that of 120 min and 180 min, a considerable decrease of hydrolysis was observed with increasing contact time above 60 min (Figure 5). This shows that, the hydrolysis time has a negative effect on the steam-alkali hydrolysis using sodium hydroxide.

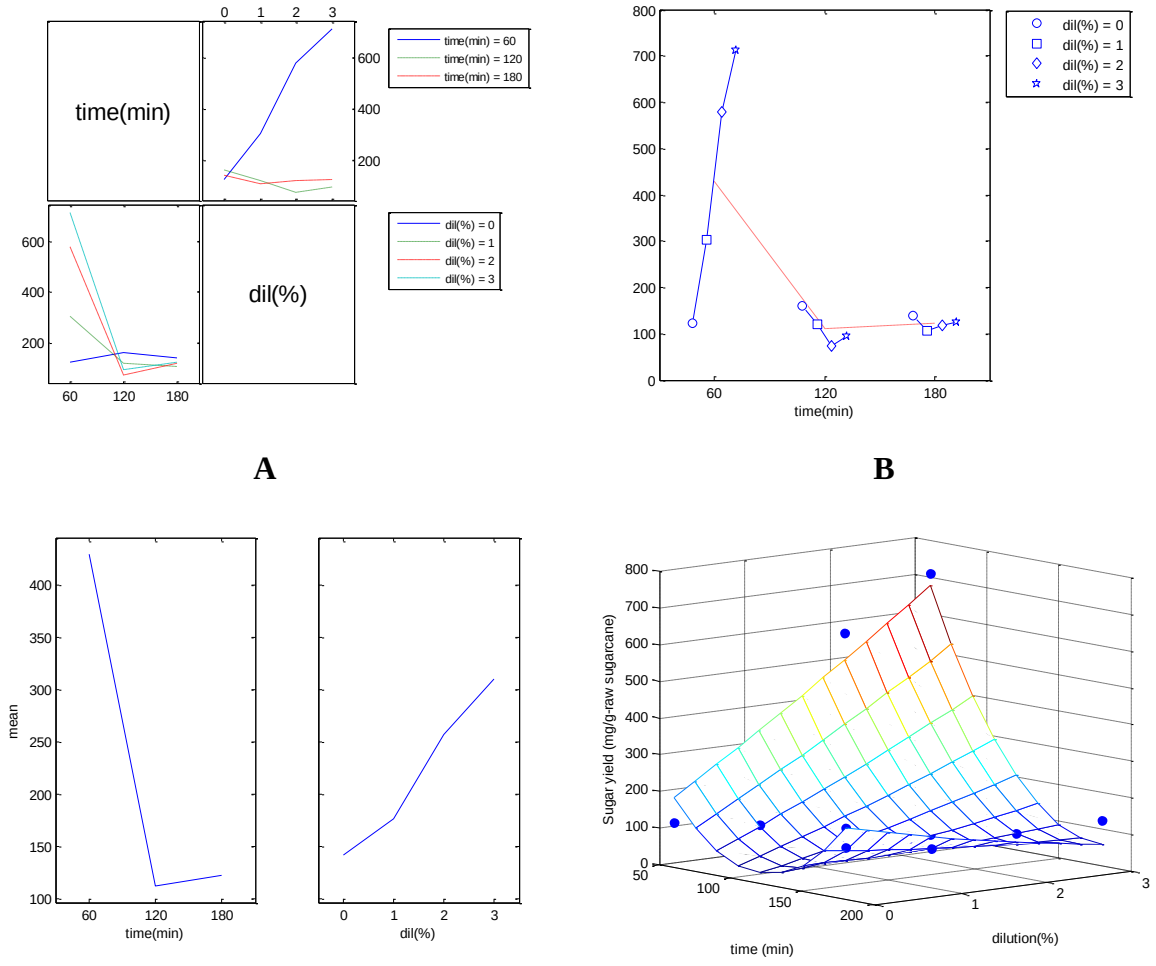


Figure 5. Interaction of hydrolysis time and sodium hydroxide concentration on the sugar yield from bagasse. (A) Interaction plot, (B) Multi-vari chart, (C) Main effects plot and (D) 3D surface plot.

Sugar yields of 0.63 g/L glucose and 1.55 g/L xylose were obtained with a pre-treatment condition of 2% NaOH for 60 mins. The mean sugar yield increased from 141.0 mg/g (neutral solution) to 310.3 mg/g when the NaOH content was set to a 3% concentration, respectively. The amount of reduced sugar was detected to be in a linear agreement with the percentage of dilution. A linear relationship was observed between the NaOH concentration and sugar yield. From the experimental results, it was concluded that steam-alkali hydrolysis with 3% NaOH for 60 min provided the highest reduced sugar of 712.4 mg/g (mainly ribose), however the least value (73.6 mg/g) was detected at 2% NaOH for 120 min.

3.3.4 Steam-alkali hydrolysis using calcium hydroxide (4th experiment)

As shown in Figure 6, for 60 min contact time, the maximum SY of about 204.2 mg/g, representing a 66.3% relative enhancement compared with the control condition (without steam-alkali treatment) was achieved for steam hydrolysis using 3% $Ca(OH)_2$. Our findings showed that increasing the hydrolysis period could decline MSY by about 58.4 – 67.4 %. This was noted when comparing the MSY of 174.6 mg/g yielded at a reaction time of 60 min with that of 120 – 180 min. Moreover, mean sugar yield of the control had declined from 141.0 mg/g to 116.7 mg/g at an $Ca(OH)_2$ of 1%. However, a relatively constant MSY (130.5 mg/g) was observed as the $Ca(OH)_2$ concentration reached 2%.

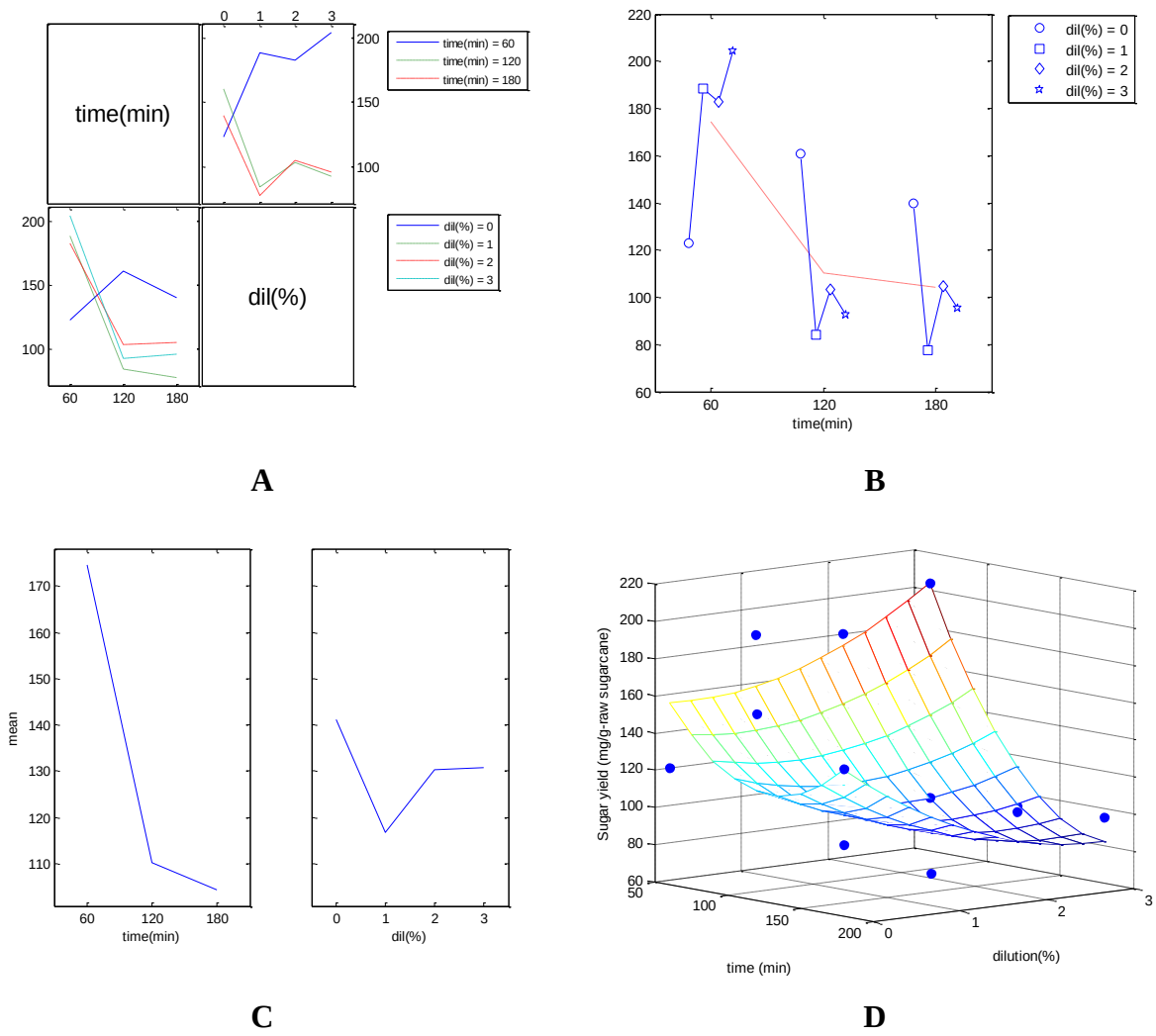


Figure 6. Interaction of hydrolysis time and calcium hydroxide concentration on the sugar yield from bagasse. (A) Interaction plot, (B) Multi-vari chart, (C) Main effects plot and (D) 3D surface plot.

Glucose and xylose yields of 0.36 g/L and 0.53 g/L, respectively were obtained with a pre-treatment of 2% Ca(OH)2 for 180 min. The optimum conditions for steam explosion using alkali treatment were found by using NaOH (i.e. 3% dilution for 60 min). The highest sugar yield obtained under Ca(OH)2 hydrolysis, for steam-alkali treatment, was lower than that of NaOH, implying that NaOH could hydrolyse the hemicelluloses in sugarcane bagasse better than Ca(OH)2. However, the lowest sugar yields for steam-alkali hydrolysis were detected when 2% NaOH was used during 120 min (mainly ribose), as well as 1% Ca(OH)2 within 180 min reaction times. As listed in Appendix one, Table 11, the correlation coefficient (R2), between measured data and simulated results from surface response methodology, of steam-alkali using NaOH and Ca(OH)2 were 0.88 and 0.72, respectively. Results of response

surface for steam-alkali revealed that, the conditions providing the least SY can be 3.1% $Ca(OH)_2$ for 184.9 min. However, the function *fminsearch* was unable to predict the optimum conditions due to the absence of function local maximum.

3.3.5 Microwave-acid hydrolysis using sulphuric acid (5th experiment)

The concentration of sulphuric acid and exposure time was varied from 0% to 1% and from 15 to 45 min, respectively during microwave-acid treatment. As shown in Figure 7, the MSY decreased from 127.6 mg/g (control) to 108.1 mg/g when the concentration was increased from 0% to 0.25% H_2SO_4. However, MSY increased gradually with further increase in acid concentration, where it recorded 142.4, 134.6 and 165.8 mg/g at 0.5%, 0.75% and 1% H_2SO_4.

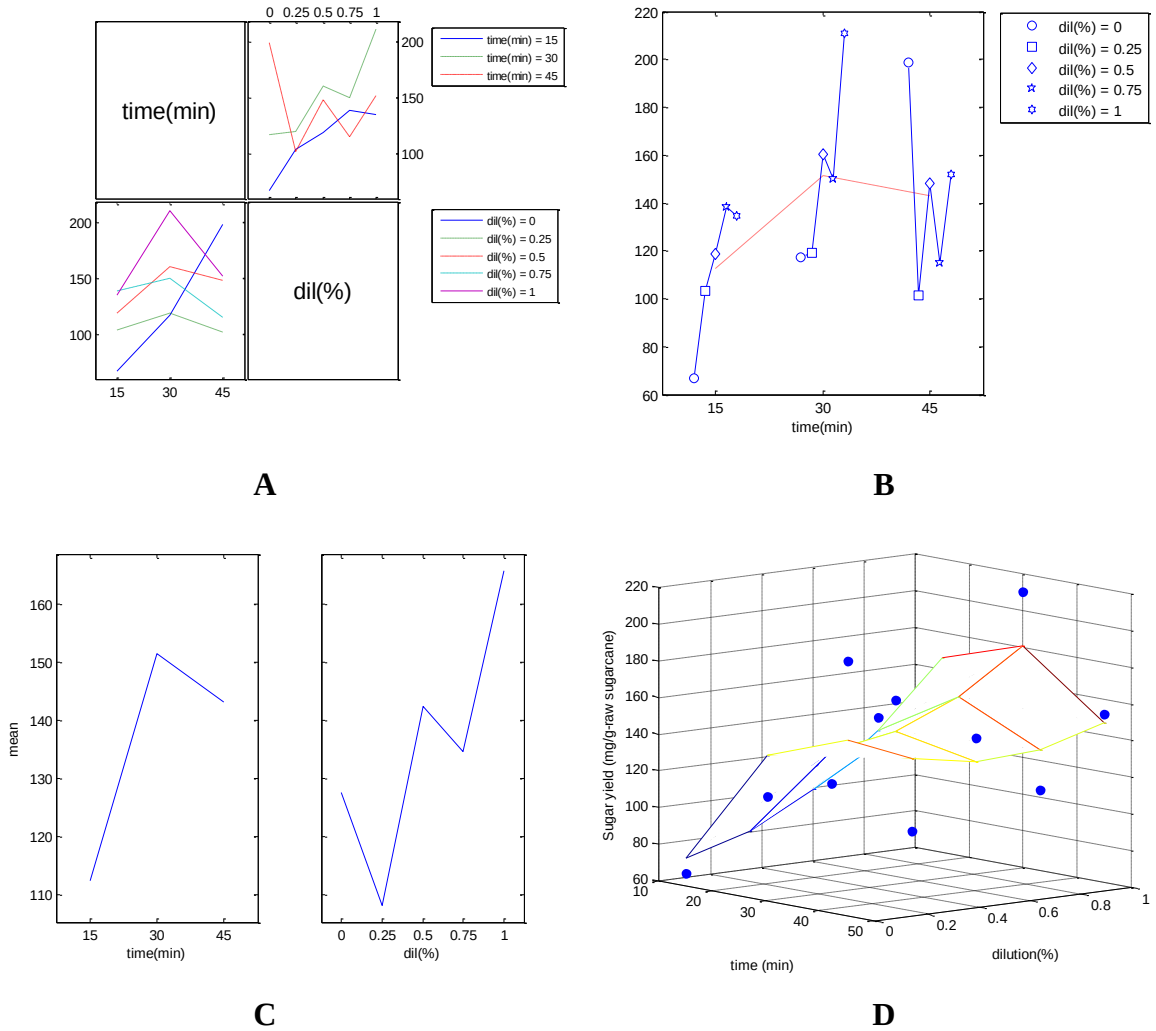


Figure 7. Interaction of hydrolysis time and sulphuric acid concentration on the sugar yield from bagasse. (A) Interaction plot, (B) Multi-vari chart, (C) Main effects plot and (D) 3D surface plot.

Previous studies revealed that, acid pre-treatments can remove a small part (but not significant) of the lignin fraction, in addition to the hemicelluloses (Kumar *et al.*, 2009). A significant increase of MSY from 112.4 mg/g at 15 min to 151.5 mg/g at 30 min was noticed during acid microwave hydrolysis using sulphuric acid, with a further decline to 143.1 mg/g at 45 min. Additionally, the maximum reduced sugar of 210.7 mg/g was noticed at 1% H_2SO_4 at 30 min reaction time. Therefore, it can be concluded that maximum reduced sugar yield obtained by microwave-acid hydrolysis could be achieved at 1% H_2SO_4 for 30 min, with fractions of 0.42 g/L (glucose) and 0.64 g/L (xylose). The minimum yield found 0.25% H_2SO_4 for 45 min.

3.3.6 Microwave-acid hydrolysis using hydrochloric acid (6th experiment)

Results obtained from microwave-acid hydrolysis (Figure 8) shows that, the maximum MSY (148.4 mg/g) was shown at 0.5% HCl representing 38% and 20.7% improvement when compared to the control and 1% HCl, respectively. These results demonstrate that the effect of increasing the acid concentration by 0.5% on hydrolysis of bagasse. At 0.5% HCl, the glucose and xylose contents were 0.23 g/L and 0.35 g/L, 0.42 g/L and 0.40 g/L, and 0.46 g/L and 0.63 g/L for hydrolysis durations of 15 min, 30 min, and 45 min. A further increasing concentration (above 0.5%) up-to 1%, leads to a 1.2-fold decrease in reducing sugar (i.e. from 148.4 mg/g to 122.9 mg/g).

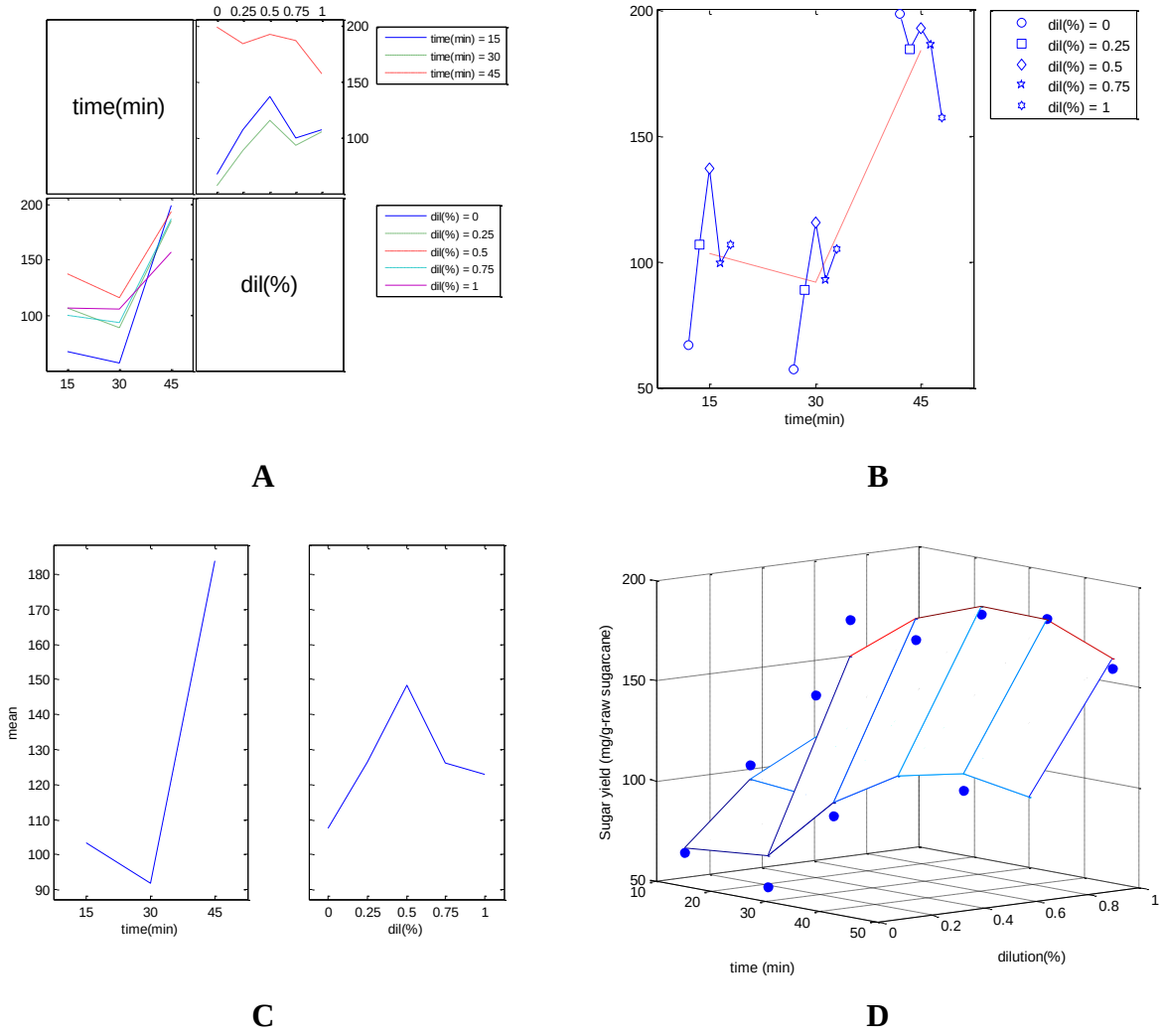


Figure 8. Interaction of hydrolysis time and hydrochloric acid concentration on the sugar yield from bagasse. (A) Interaction plot, (B) Multi-vari chart, (C) Main effects plot and (D) 3D surface plot.

This shows the significant negative effects of microwave hydrolysis by hydrochloric acid at relatively high concentration. Therefore, with regard to acid microwave treatment using HCl, the optimum concentration was 0.5% for 15 – 45 min. It can be concluded that microwave-acid treatment was optimal at 1% H_2SO_4 for 30 min, as well as 0.5% HCl for 45 min. Appendix one, Table 12, shows the correlation between measured data and simulated results from surface response methodology has R^2–value of 0.59 and 0.92 for microwave-acid using H_2SO_4 and HCl, respectively. The function *fminsearch* failed to predict the conditions that would minimize and/or maximize the SY. This was due to *fminsearch* only being able to provide local solutions, which were absent from the current surface response.

3.3.7 Microwave-alkali treatment using sodium hydroxide (7th experiment)

The mean sugar yield decreased from 122.0 mg/g (control) to 73.3 mg/g, with an increasing concentration to 1% NaOH (Figure 9). However, the MSY remained relatively constant (70.7 mg/g) with 2% NaOH.

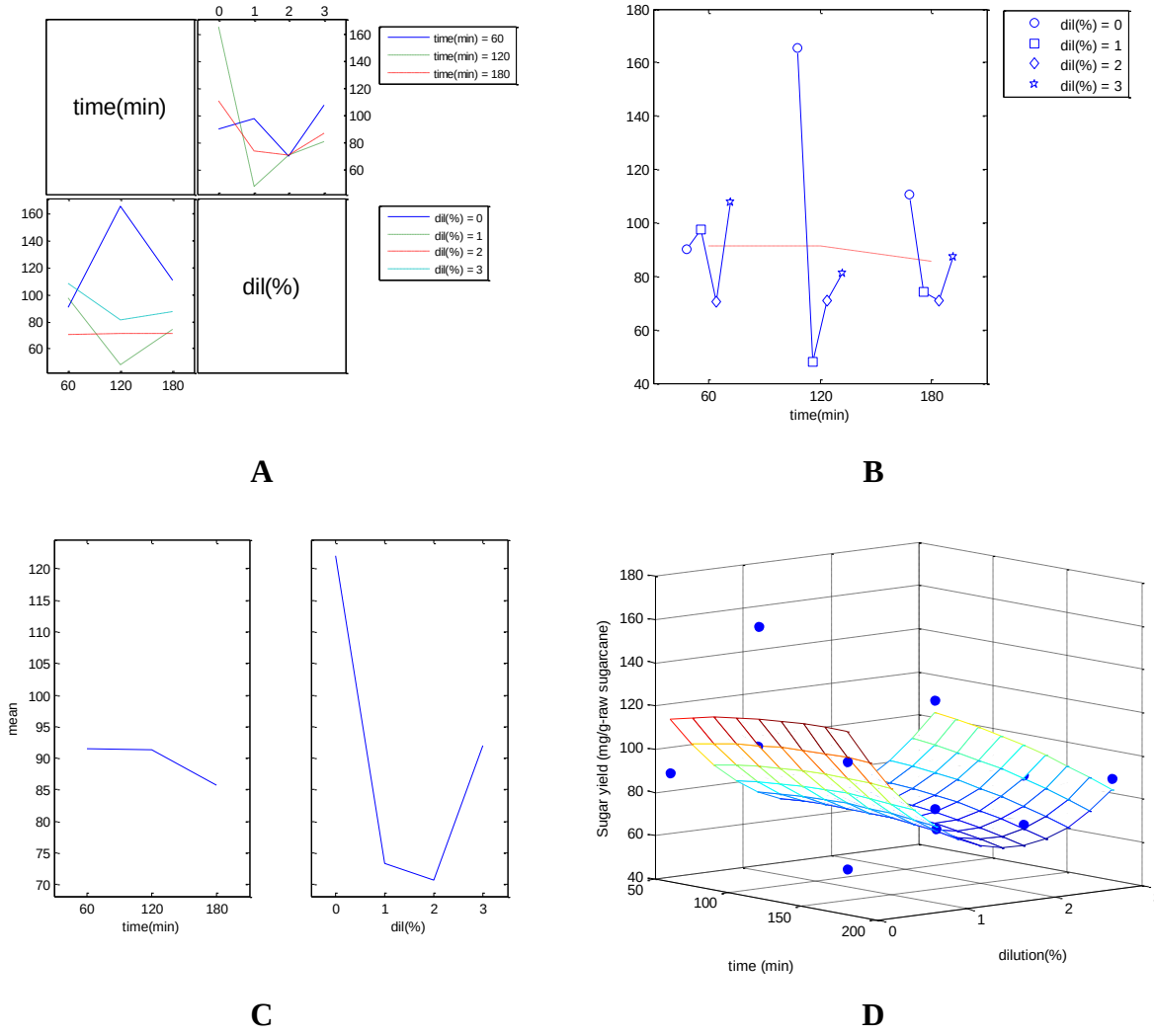


Figure 9. Interaction of hydrolysis time and sodium hydroxide concentration on the sugar yield from bagasse. (A) Interaction plot, (B) Multi-vari chart, (C) Main effects plot and (D) 3D surface plot.

Thereafter, MSY was raised again to 92.0 mg/g with a further rise in dilution to 3% NaOH. The effect of reaction time on MSY was different, as it remained relatively stable (91.4 mg/g) during (60 – 120 min). Thereafter, an increase in time showed a slight depletion of bagasse hydrolysis dropping the MSY to 85.7 mg/g at 180 min. These results indicated that the

maximum reduced sugar during microwave alkali hydrolysis using NaOH was denoted at 3% for 60 min, with 0.11 g/L (glucose) and 0.02 g/L ribose release.

3.3.8 Microwave-alkali treatment using calcium hydroxide (8th experiment)

From Figure 10, the MSY was observed to increase from 98.3 mg/g to 163.4 mg/g with an increase in hydrolysis time from 60 min to 120 min, after which retarded to 102.9 mg/g with a further increase in of time to 180 min. The concentration had a profound positive impact on hydrolysis of bagasse, as 1% $Ca(OH)_2$ resulted in 1.2-fold more sugar yield than that of the control (Conc. 0%).

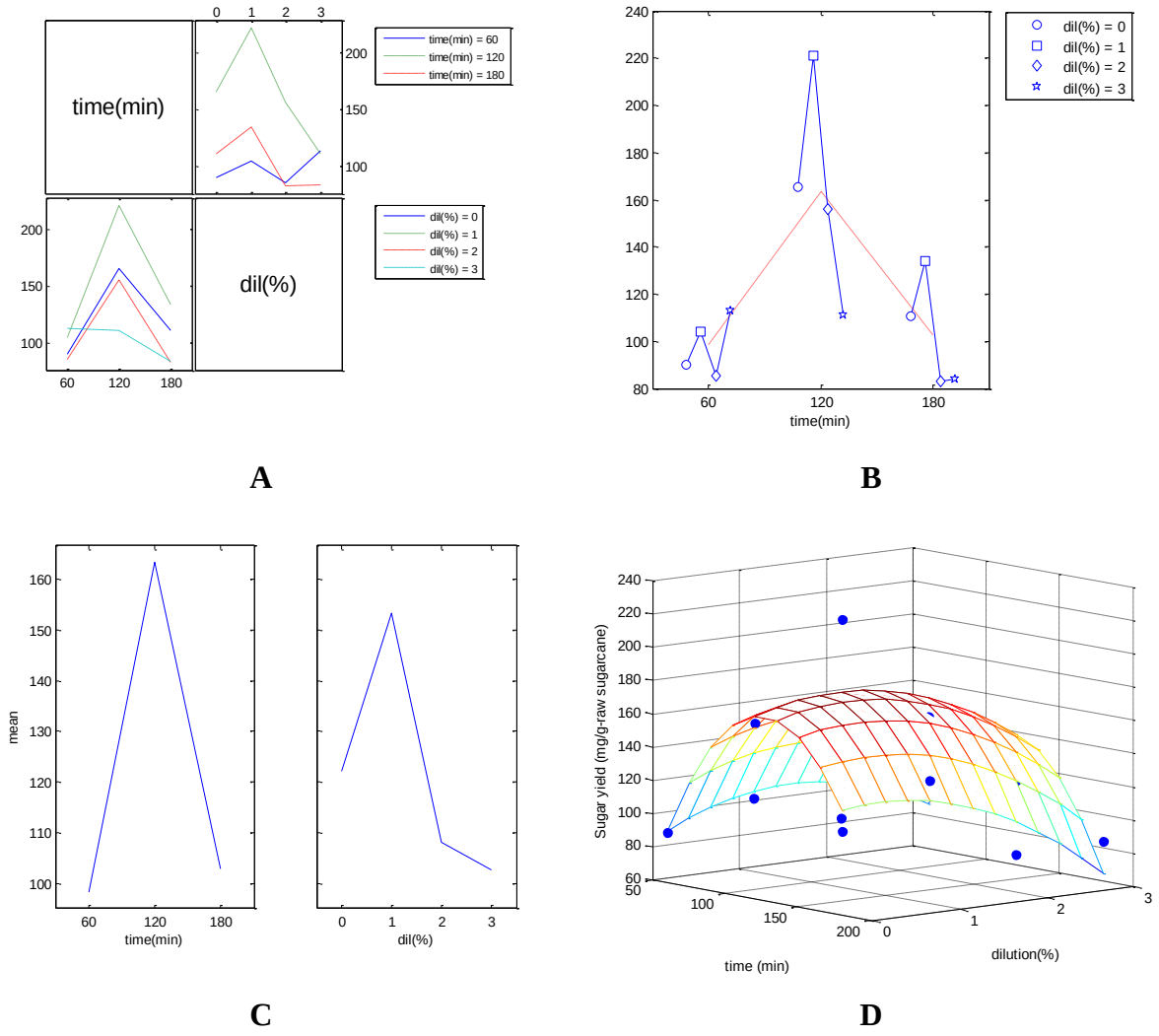


Figure 10. Interaction of hydrolysis time and calcium hydroxide concentration on the sugar yield from bagasse. (A) Interaction plot, (B) Multi-vari chart, (C) Main effects plot and (D) 3D surface plot.

A subsequent increase in concentration to 2% and 3% lead to a decrease in MSY to 108.1 mg/g and 102.7 mg/g. The maximum reduced sugar of 221.2 mg/g, corresponding to 33.7% relative enhancement, was attained at a concentration of 1% $Ca(OH)_2$ at 120 min. For microwave-alkali treatment, the maximum reduced sugar yields from reaction times 60 min, 120 min and 180 min was detected at $Ca(OH)_2$ concentrations of 3%, 1% and 1%, respectively. Using results of surface response methodology in Appendix one, Table 13, the R^2–value between measured and predicted data of microwave-alkali using NaOH and $Ca(OH)_2$ were 0.55 and 0.74, respectively. By applying function *fminsearch*, the optimum conditions for microwave-alkali hydrolysis was 0.91% $Ca(OH)_2$ for 123.7 min. After hydrolysis of hemicelluloses, percentages of sugar contents for glucose and xylose were 0.38 g/L and 0.35 g/L (60 min 3% $Ca(OH)_2$), 0.15 g/L and 0.00 g/L (120 min 1% $Ca(OH)_2$), and 0.16 g/L and 0.00 g/L (180 min 1% $Ca(OH)_2$).

High pressure liquid chromatography (HPLC) was used to analyse the individual sugar composition in the hydrolysed samples that demonstrated high carbohydrate content when analysed by phenol sulfuric acid method. Appendix one Table 14, shows the soluble fraction of sugarcane bagasse hydrolysed at different conditions, being steam-acid, steam-alkali, microwave-acid and microwave-alkali. In all the treatments performed, xylose and glucose were the most prominent sugars present in the hydrolysate; which were the two sugars of interest. Further, steam-acid treatment using 0.5% H_2SO_4 for 60 min was the optimum for the release of xylose (2.329 g/L) and glucose (1.049 g/L) from SCB. The current results can be related to other findings that favour these reaction conditions (Candido *et al.*, 2012, Chen *et al.*, 2011, Manzoor *et al.*, 2012, Jackson De Moraes Rocha *et al.*, 2011), a low xylose and high glucose concentration is preferred when choosing the substrate of interest. High xylose content can degrade and produce inhibitors during fermentation (furfural) whilst high glucose content will be ideal for the growth of most microorganisms (Sambusiti *et al.*, 2013).

Observations of the current study could be compared to the earlier reports. Fuentes (Fuentes *et al.*, 2011) have reported that upon sugarcane bagasse pre-treatment with lime, it was found that the maximum yield of glucose was 228.45 mg/ g-raw biomass, corresponding to 409.9 mg/ g-raw biomass of total reducing sugars, with the pre-treatment performed at 90°C, for 90 h, and with a lime loading of 0.4 g/ g-dry biomass.

Additionally (Rodrigues *et al.*, 2011) observed that, after enzymatic hydrolysis, alkali concentration had an influence on glucose formation, after pre-treatment with 0.2 and 1.0 mol/L of NaOH (372 ± 12 and 355 ± 37 mg/g-glucan) when 2% (w/v) of cashew apple bagasse pre-treated by microwave-assisted alkali pre-treatment was used (Rodrigues *et al.*, 2011). Furthermore, researchers observed that microwave treatment (600 W) of sugarcane bagasse using 1% NaOH for 4 min coupled with enzymatic hydrolysis produced a reducing SY of 0.665 g/g dry biomass, while joined microwave-alkali-acid treatment with 1% NaOH followed by 1% sulfuric acid, the reducing SY increased to 0.83 g/g dry biomass. However, with microwave-acid, maximum reducing SY (0.249 g/g) was noted at 100 W for 22 min treatment time (Binod *et al.*, 2012).

In another study, two different dilute acid pre-treatments (sulfuric and hydrochloric acid) and one mixture of these acids were for tested with sweet sorghum bagasse. They liberated that the three different pre-treatments yielded similar amounts of total potentially fermentable sugars (390 – 415 mg-sugar/g-bagasse). However, the HCl treatment was a suitable option due to its comparatively lower hydrolysis time and acceptable generation of C5 and C6 fermentable sugars (Heredia-Olea *et al.*, 2012).

3.3.10 Evaluation of morphological changes of sugarcane bagasse during pre-treatment using scanning electron microscopy

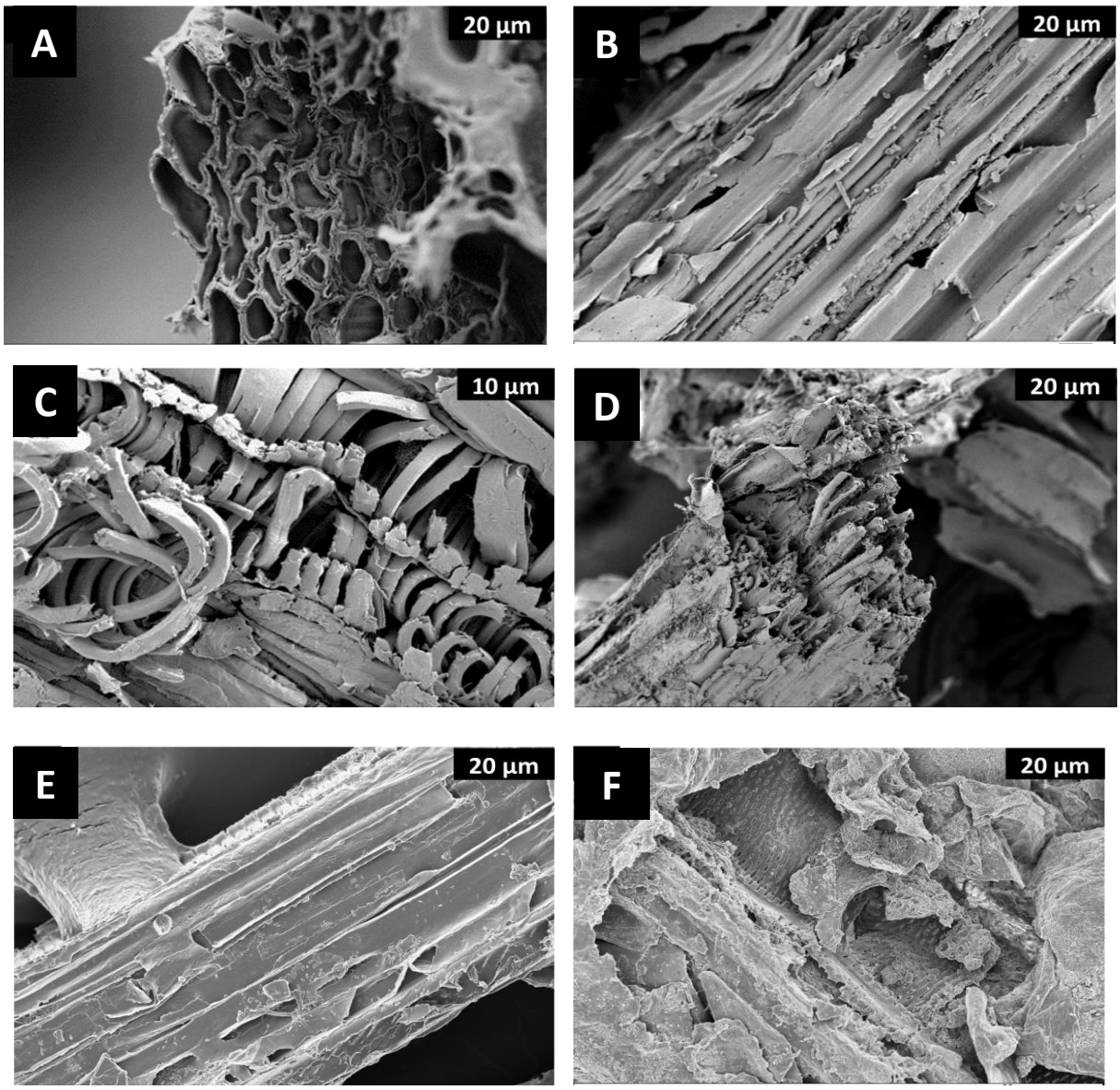


Figure 11. Morphological characteristics of bagasse before and after treatment using steam and microwave hydrolysis

A. Native bagasse fibres; B. Steam treated (30 min) 0.25% HCL; C. Steam treated (60 min) 0.25% H_2SO_4; D. Steam treated (180 min) 2% $Ca(OH)_2$; E. Microwave treated (30 min) 1% H_2SO_4; F. Microwave treated (120 min) 1% $Ca(OH)_2$

A panoramic micrograph of the sugarcane bagasse structure was investigated using SEM. The physical changes occurred during pre-treatment are shown in Figure 11, and can be compared to preceding studies (Monlau *et al.*, 2013, Sinha and Pandey, 2013). Before treatment, a general view of woody fibres and a compact structure of bagasse can be seen. After undergoing steam-acid treatment, the structure of the bagasse at a reaction time of 30 min had shown slight breakages on the surface. This could be attributed to the removal of the hemicelluloses and extractives occurred during pre-treatment. However after 60 min, the entire bagasse structure was disintegrated at an acid concentration of 0.25% (sulphuric acid), exposing the inward structural components, causing the formation of a bundle of fibres. After steam-alkali treatment, tiny holes were pronounced on the surface, whilst some images for calcium hydroxide treated bagasse had residue embedded on the surface. Microwave pre-treated samples had shown a much compact structure after 30 min of acid treatment, whilst alkali treatment had exposed much more of the plant structure.

3.4 Conclusions

- Steam assisted hydrolysis of SCB using 0.5% H_2SO_4 and 0.25% HCl was adequate to extract the sugars.
- Hydrolysis time has a negative effect on the steam-alkali hydrolysis using NaOH (for 60 – 180 min).
- The highest MSY obtained under steam-NaOH hydrolysis was 2.5-fold higher than that under steam-$Ca(OH)_2$.
- Steam-NaOH detected MSY results equivalent to 1.2-fold and 1.3-fold higher than steam-acid using H_2SO_4 and HCl, respectively.
- Increasing exposure time higher than 6 min has no effect on SCB hydrolysis when using aqueous NaOH system under microwave irradiation.
- The optimal irradiation time during using microwave hydrolysis for 1 - 3% $Ca(OH)_2$ was 12 min.
- SEM was effective in distinguishing the degree of structural damage after every treatment.

CHAPTER FOUR: EVALUATION OF THERMAL PRE-TREATMENT METHODS ON SEWAGE SLUDGE TO ELIMINATE HYDROGEN CONSUMING MICROORGANISMS AND TO SELECT HYDROGEN PRODUCING BACTERIA.

4.1 Introduction

Selection of "ideal" microbial consortium is a critical step in the dark fermentation process (Wang and Wan, 2008c, Lin *et al.*, 2012). The hydrogen production efficiency however is highly dependent on the anaerobic bacteria (pure or mixed) present in the system (Elsharnouby *et al.*, 2013). Conditions that promote high hydrogen yields can be monitored closely and precisely in pure culture systems, however full scale application of pure cultures is considered unfavorable due to strict process control, medium sterilization and fewer choices of feedstock. Therefore, use of mixed cultures is favoured and regarded as feasible for large scale production of hydrogen (Ntaikou *et al.*, 2010). By using a mixed culture system, operation is much easier and a broad selection of feedstock can be incorporated into the digestion process (Li and Fang, 2007).

Anaerobic sludge is often used as an ideal consortium for hydrogen production, due to the presence of higher diversity of hydrogen producing bacteria. However, presence of large numbers of hydrogen consuming bacteria such as methanogens is the main challenge when sewage sludge is used as the seed inoculum. In recent past, various pre-treatment methods were employed to inactivate the undesirable microbes found in the inoculum sludge simultaneously protecting the hydrogen producing bacterial activity (Cai *et al.*, 2004).

Thermal treatment is one of the most widely employed treatment method to eliminate hydrogen consuming and other undesirable microbes from the sludge (Liang *et al.*, 2008, Mu *et al.*, 2007, Yu *et al.*, 2013). During thermal treatment, the non-spore forming hydrogen producing bacteria are expected to be inactivated by heat shock treatment, whereas the spore forming hydrogen producing bacteria are expected to bypass heat shock by forming highly heat resistant spores.

Although thermal pre-treatment is been employed by most researchers, this method also has the limitation of reducing desired microbial diversities which warrants the requirement of

further optimization of this technique for eliminating undesired microbes while retaining the maximum desirable microbial community.

The main goal of this study was therefore to compare heat and sterilization pre-treatment to quantitatively and monitor the change in microbial community at different time intervals using the molecular based techniques (Ren *et al.*, 2008, Wang *et al.*, 2011). The microbial community structure was analysed by Fluorescent in-situ hybridization (FISH) to observe specific groups of bacteria from treated and untreated anaerobic sludge. FISH was a useful tool to accurately analyse progressive changes of target organisms (Lee *et al.*, 2009b).

4.2 Materials and method

4.2.1 Inoculum sludge

The inoculum sludge samples used in this study was obtained from the anaerobic tank of two local municipal wastewater treatment plants (Kingsburgh and Northern WWTP), treating domestic and industrial wastes respectively.
Characterization of collected sludge was carried out according to the standard methods (Table 3). Factors such as pH, total solid (TS), total suspended solids (TSS) and volatile suspended solids (VSS) were determined following the protocol of (American Public Health *et al.*, 1998). Whereas ammonia, nitrite, nitrate and phosphorus were analysed by colorimetric method using Thermo Scientific Gallery Photometric automated analyser.

4.2.2 Thermal Sludge pre-treatment

Thermal pre-treatment was used to assess its ability to eliminate methanogenic hydrogen consuming bacteria within the sludge samples using an autoclave and boiling treatment methods.

All the experiments were performed in batch mode using one litre screw cap glass bottles.

In sterilization pre-treatment, the anaerobic sludge was subjected to 121°C at 1.4 kg/cm (Speedy autoclave, HL-341) and in heat shock pre-treatment, sludge was boiled at 102°C in an oven (Incotherm, economy). Similarly, control experiments were operated in parallel containing anaerobic inoculum at room temperature. The reactions were performed in triplicate at time intervals of 30 and 60 minutes. Each time interval had an individual arrangement of bottles used for single analysis to maintain anaerobic conditions of samples being used for microbial analysis.

4.2.3 Microscopy and quantification

The microbial community analysis of both wastewater treatment plants were determined by light and fluorescent microscopy. Gram staining was used to examine the morphological characteristics of the sludge samples using a light microscope. Fluorescent in-situ hybridization was the technique used to quantitatively identify microorganisms by targeting complementary Deoxyribonucleic acid (DNA) sequences with oligonucleotide probes labelled at the 5'- end with 6-carboxy-fluorescein (FAM). Table 2 indicates the microbial groups of interest and their hybridization condition.

4.2.3.1 Light Microscopy (Gram staining)

Smears of test sample had been prepared on a glass microscope slide, the sample was left to air dry and thereafter fixed by passing the slide through a flame. The dried slide was stained with crystal violet reagent for a minute and the excess stain was rinsed off with tap water. Grams iodine was then added for a minute and rinsed off with tap water. The slides were thereafter dehydrated by the addition of 95% ethanol for 30 seconds. The 95% ethanol was washed off with tap water and the slide was then counter stained with safranin reagent for 1 minute. After a minute the slide was washed with tap water and blot dried with paper towel. The slide was then viewed with a Nikon eclipse 80i light microscope under oil immersion (Csuros, 1999).

4.2.3.2 Epifluorescence Microscopy

Sample fixation: Two millilitres of sludge samples were centrifuged for 3 minutes at 3000 rpm (Heraeus Biofuge, Fresco). The supernatant was discarded and the pellet was resuspended with a 1ml of 1× phosphate buffer solution, this procedure was repeated 3 times. Thereafter, the supernatant was removed and the pellet was resuspended with 750µl of 4% paraformaldehyde and 250µl of 1× phosphate buffer solution. The samples were then left overnight at 4°C. After the incubation period, the samples were stored in 500µl of 100% ethanol and 500µl of 1× phosphate buffer until further use.

Hybridization: The fixed samples were spotted onto poly-L-lysine coated microscope slides, air dried at room temperature for 15 minutes and dehydrated in 50%, 80% and 100% ethanol (vol/vol) for 3 minutes in each dilution. Samples were then hybridized with 9µl of hybridization solution containing 1.8 M NaCl, 1M Tris-HCl (pH 7.2), 10% sodium dodecyl sulfate (SDS), formamide and 1µl of specific oligonucleotide probe (Table 2). The slides were placed into 50ml polyethylene tubes with hybridization buffer and incubated overnight at 46°C. The slides were thereafter washed with pre-warmed (48°C) washing solution (1.8 M NaCl, 1M Tris-HCl (pH 8), 10% SDS) and incubated at 48°C for 30 minutes (Hybridization oven, Stovall HO-10).

Microscopy and imaging: To determine total cell counts, slides were rinsed with distilled water and counterstained with 10µl of 4',6-diamidino-2-phenylindole (DAPI) for 3 minutes. Slides were rinsed with distilled water, air dried in the dark at room temperature. Vectorshield (antifade solution) was used to mount slides, followed by a slide cover slip and then viewed using Zeiss, Axiolab epifluorescent microscope. FAM fluorochromes were added to the oligonucleotide probes to distinguish the hybridized cells from the total number of cells by excitation at a wavelength of 518nm whilst DAPI staining was used to determine the total number of cells at a excitation wavelength of 345nm. Thereafter, images captured were imported to AxioVision Viewer 4.8 for cell enumeration (Seviour and Nielsen, 2010, Amann *et al.*, 1990).

Table 2. FAM labeled oligonucleotide probes used in this study

Probe name	**Target group**	**Probe sequence (5'-3')**	**Formamide (%)**	**Reference**
Alf 1B	Alphaproteobacteria	CGTTCGYTCTGAGCCAG	20	(Manz W., 1992)
BET 42a	Betaproteobacteria	GCCTTCCCACTTCGTTT	35	(Manz W., 1992)
GAM 42a	Gammaproteobacteria	GCCTTCCCACATCGTTT	35	(Manz W., 1992)
EURY 498	Euryarchaeota	CTTGCCCRGCCCTT	0	(Burggraf *et al.*, 1994)
CHIS 150	*Clostridium*	TTATGCGGTATTAATCTYCCTTT	0	(Franks *et al.*, 1998)
EBAC 1790	Enterobacteriaceae	CGTGTTTGCACAGTGCTG	40	(Bohnert *et al.*, 2000)
EUB 338	most Bacteria	GCTGCCTCCCGTAGGAGT	35	(Amann *et al.*, 1990)
EUB 338II	Planctomycetales	GCAGCCACCCGTAGGTGT	35	(Daims *et al.*, 1999)
EUB 338III	Verrucomicrobiales	GCTGCCACCCGTAGGTGT	35	(Daims *et al.*, 1999)
LGC 0355	Firmicutes (Low G+C gram-positive bacteria)	GGAAGATTCCCTACTGCTG	20	(Hallberg *et al.*, 2006)

4.3 Results and discussion

4.3.1 Sludge characterization

Sludge samples collected from each wastewater treatment plant (WWTP) were compared by primary assessment to determine the most suitable sludge composition that was used for treatment in this study.

Table 3. Characteristics of untreated sludge from both wastewater plants

Parameter	Untreated sludge	
	Northern Wastewater Works	Kingsburgh Wastewater Treatment Plant
pH	7.1	6.67
TS (mg/L)	6911.3	3230.0
TSS (mg/L)	5734.7	3141.7
VSS (mg/L)	4371.3	2648.3
Ammonia (mg/L)	1.94	25.36
Nitrite (mg/L)	5.05	0.04
Nitrate (mg/L)	3.46	1.6
Phosphorus (mg/L)	229.47	61.44

Fluorescent in-situ hybridization was used to detect and quantify the microbial consortium of the samples (Figure 12 and 13). Ten oligonucleotide probes were selected in this study to identify the major hydrogen producing microorganism as well as hydrogen consuming methanogens in the sludge. Probes used in this study were chosen according to taxonomic ranking as follows: targeting the Bacterial domain (EUB 338, EUB 338II, EUB 338III mix), phylum of Proteobacteria and classes of Alpha, Beta and Gamma (Alf 1B, BET 42a, GAM 42a). Phylum of Firmicutes was used to represent the classes of Bacilli and Clostridia (LGC 0355). At a family and genus level Enterobacteriaceae (EBAC 1790) and *Clostridium* (CHIS150) respectively, was used to classify the presence of hydrogen producing bacteria. From the Archaea domain, the phylum Euryarchaeota, probe EURY498 was used to observe the methanogenic microorganisms.

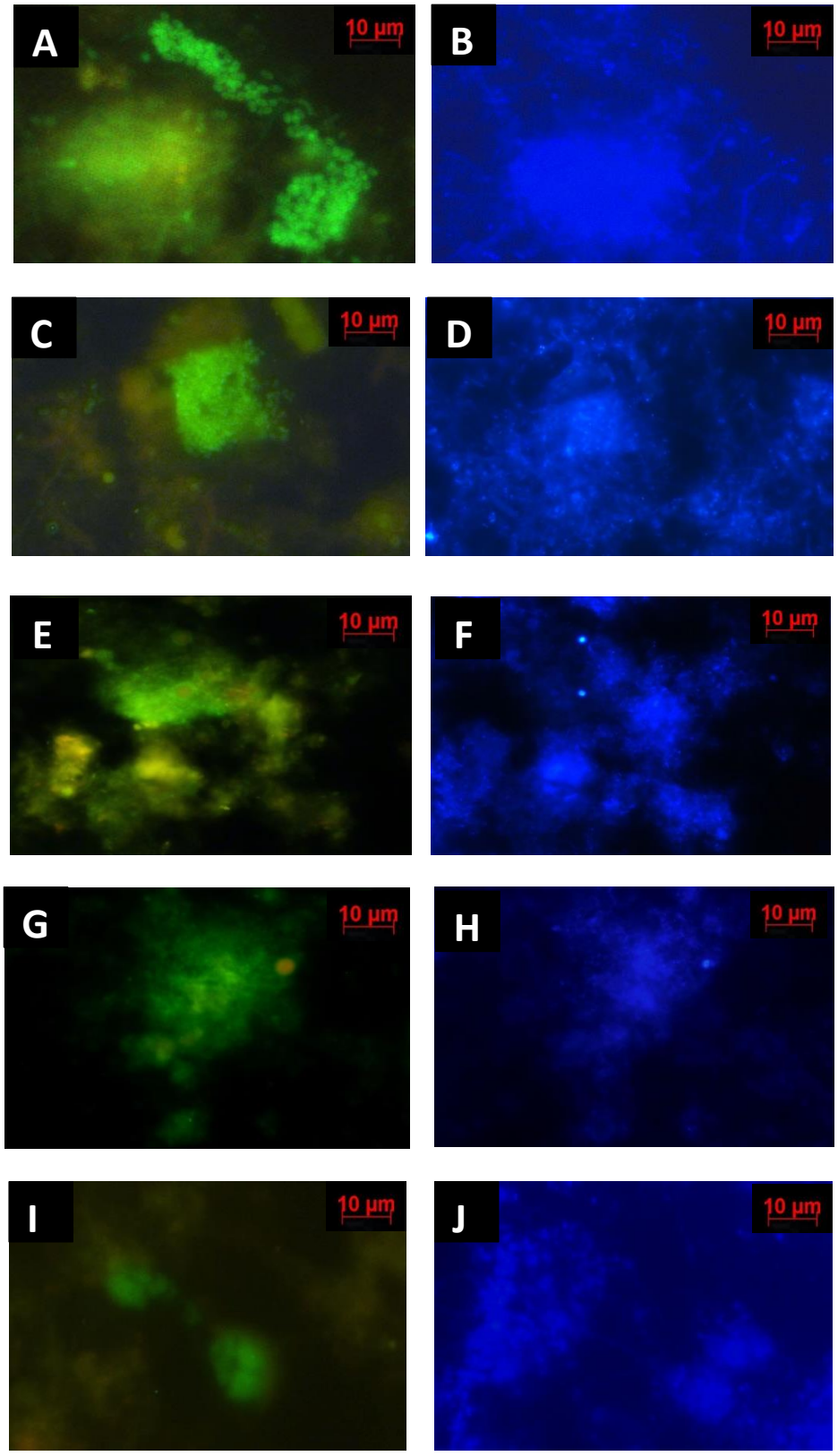


Figure 12. FISH images showing hybridization of specific oligonucleotide probes and DAPI stained images in the study

A & B- Bacterial domain (EUB 338, EUB 338II, EUB 338III mix); C & D- Alphaproteobacteria; E & F -Betaproteobacteria; G & H- Gammaproteobacteria and I & J- Firmicutes (Low G+C gram-positive bacteria).

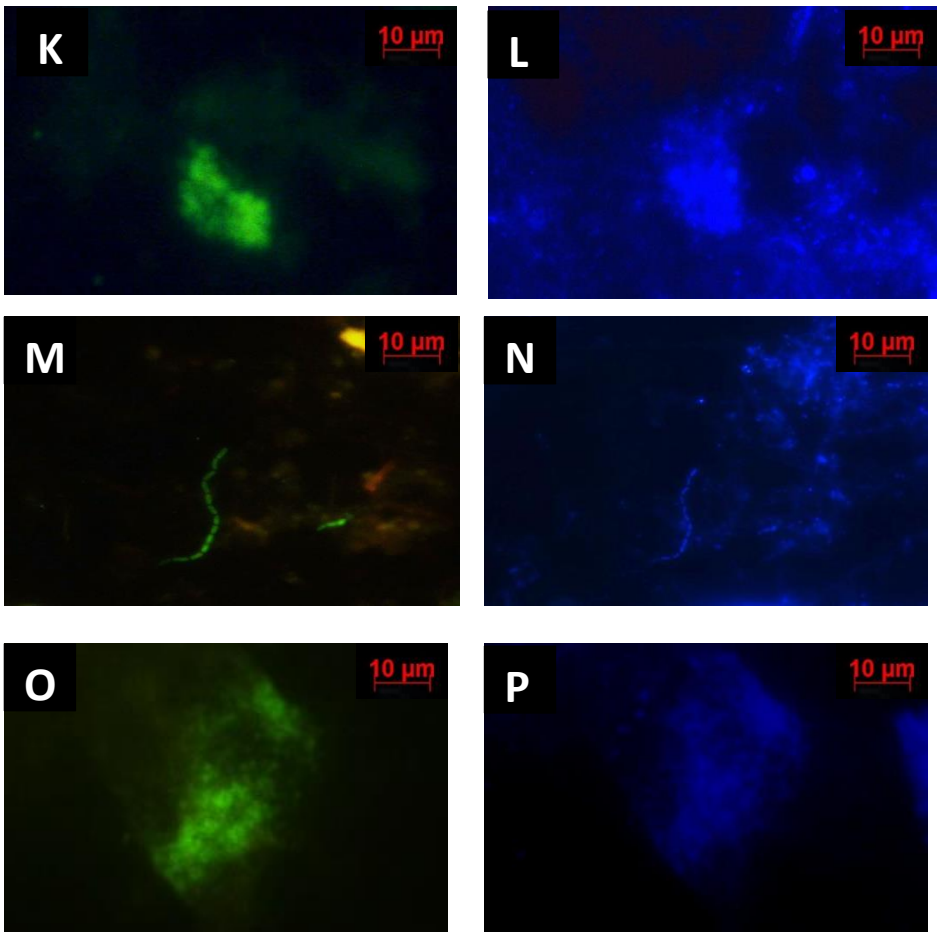


Figure 13. FISH images showing hybridization of specific oligonucleotide probes and DAPI stained images in the study

K & L- Euryarchaeota; M & N- *Clostridium* and O & P- Enterobacteriaceae.

The data collected from hybridized cells were quantified by counting the number of cells hybridized per probe against the total number of cells stained. An average of ten random images was captured to establish the overall percentage of fluorescence. The percentage of microbial communities from both WWTP was compared in Figures 14 and 15 below.

Figures 14 and 15 illustrate the percentages of specific microorganisms in each plant. From the results obtained, a much higher percentage of microbial consortiums were found in the Kingsburgh WWTW compared to the Northern WWTW. This can be attributed to the type of treatment plant. Kingsburgh WWTW treats mostly domestic waste with very low percentages of industrial effluent (5-10%) whereas the Northern WWTW treats mostly industrial effluent

which may contain toxic substances that could affect the microbial diversity (Dalzell *et al.*, 2002, Gutierrez *et al.*, 2002, Sa'idi, 2010).
Both the plants however revealed the presence of hydrogen producing and consuming microorganism. The phylum of Proteobacteria and Firmicutes were hybridized displaying several individual cells illuminated.

Further investigations using species specific probes revealed the presence of hydrogen producing *Clostridium* (1.8%) and *Enterobacteriaceae* (2.21%) in Kingsburgh WWTW. *Clostridium* belongs mostly to cluster I, hence the probe chosen in this study (CHIS150) was used to hybridize the clostridia cluster. It was found that approximately 2% of the total number of cells was methanogenic bacteria. These cells appeared in green cocci clusters.
In addition, DAPI stained images of hybridized cells, confirmed the presence of microbes on the slide therefore eliminating autoflouresence.

From the results obtained, sludge from Kingsburgh wastewater treatment plant contained an enhanced population of hydrogen producing bacteria, which would obtain an ideal hydrogen producing consortium for anaerobic fermentation. Sludge was thereafter collected from Kingsburgh wastewater treatment plant and used for experimental analysis to determine the effect of thermal pre-treatment in eliminating the presence of methane producing microorganisms, whilst still preserving the hydrogen producing population.

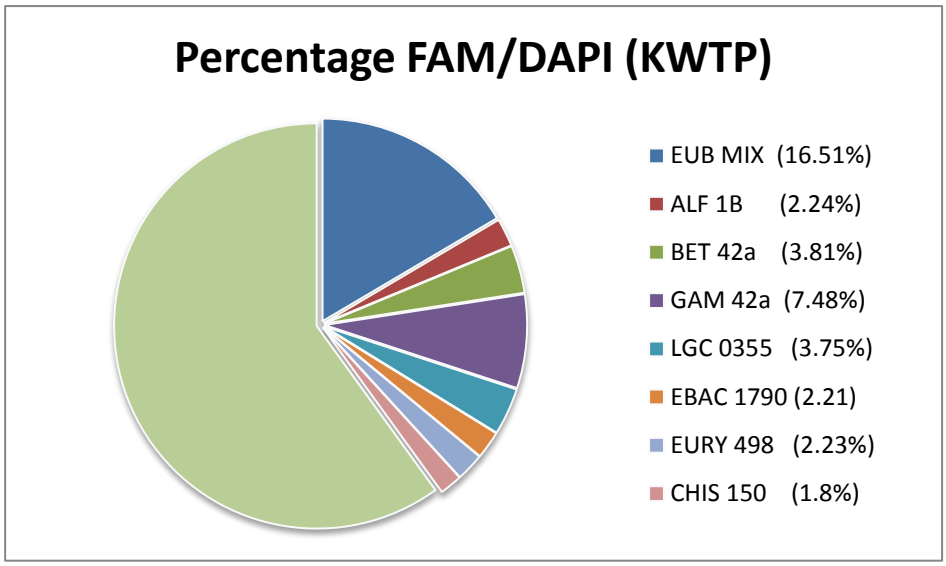


Figure 14. Representation of percentage hybridization of each oligonucleotide probe over the total number of DAPI stained cells in Kingsburgh WTP.

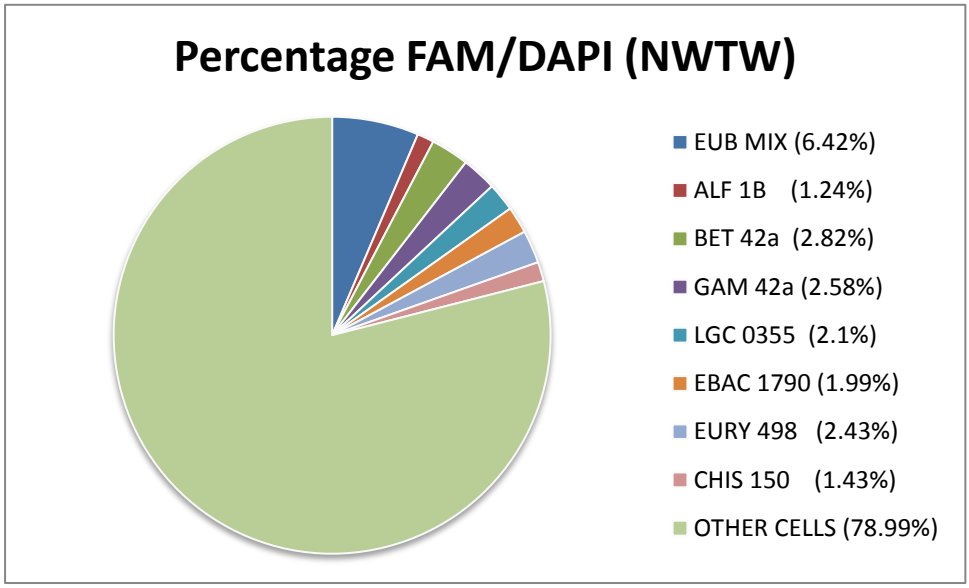


Figure 15. Representation of percentage hybridization of each oligonucleotide probe over the total number of DAPI stained cells in Northern WTP.

4.3.2 Thermal Sludge pre-treatment

Thermal treatment (autoclave and boiling) was chosen as pre-treatment methods to eliminate hydrogen consuming bacteria from the inoculum sludge. The optimization studies were conducted in batch experiments at different time intervals. Results obtained from these experiments were evaluated to understand the effect of heat treatment on the total microbial population.

The treated sludge samples were initially evaluated by gram staining to recognize the presence of spore forming hydrogen producing bacteria. Figure 16 A represent a control sludge sample taken at 0 minutes and Figure 16B was captured after 30 minutes of boiling. As shown in Figure 16, thermal treatment exhibited the formation of spore forming capabilities, represented as clear/unstained spots in the images captured. FISH technique was used to further identify and quantify the microbial population from the treated and untreated samples at different time intervals.

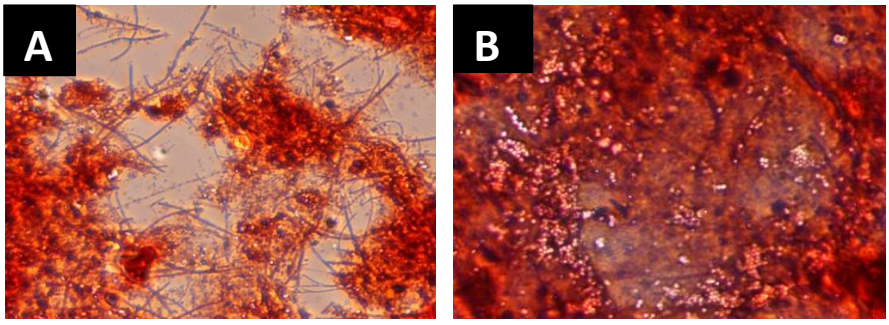


Figure 16. Image A and B are Gram stained images from KWTP before and after boiling treatment respectively.

It is apparent from Figures 17, 18 and 19 that the pre-treatment methods employed on the seed anaerobic inoculum did have a significant influence on the hybridization of specific oligonucleotide probes. The negative control samples (Figure 17) displayed similar percentages of hybridized cells after 30 and 60 minutes of sampling. These results show no significant difference in the population shift with the bacterial community (EUB mix) remaining at approximately 46% and hydrogen producing *Clostridium* and Enterobacteriaceae at approximately 1% and 2% respectively. The presence of methanogenic

bacteria was also evident in the sample as the phylum Euryarchaeota remained stable (1.93-1.83%). However, after 30 and 60 minutes of sterilization treatment (Figure 18) the only two oligonucleotide probes that exhibited hybridization was from the bacterial domain (EUB 338, EUB 338II, EUB 338III mix) and *Clostridium* (CHIS150). This indicated that treating the anaerobic sludge at 121°C completely destroyed the presence of methanogenic bacteria but also eliminated the hydrogen producing family of Enterobacteriaceae. The bacterial domain had declined by a further 19% of hybridized cells after 60 minutes of treatment whilst the population of *Clostridium* cells (3.19%) did not significantly change. However, after 30 and 60 minutes of boiling treatment (Figure 19) the bacterial domain was not at a low percentage when compared to that of sterilization treatment. Boiling at 30 minutes preserved the populations of hydrogen producing *Clostridium* (4.32%) and Enterobacteriaceae (1.81%) whilst being able to eliminate the occurrence of methanogenic bacteria. After 60 minutes of boiling treatment there was no hybridization of the Enterobacteriaceae family and a minor decline in the bacterial domain (24.69%) and *Clostridium* (3.72%) cell count was noted.

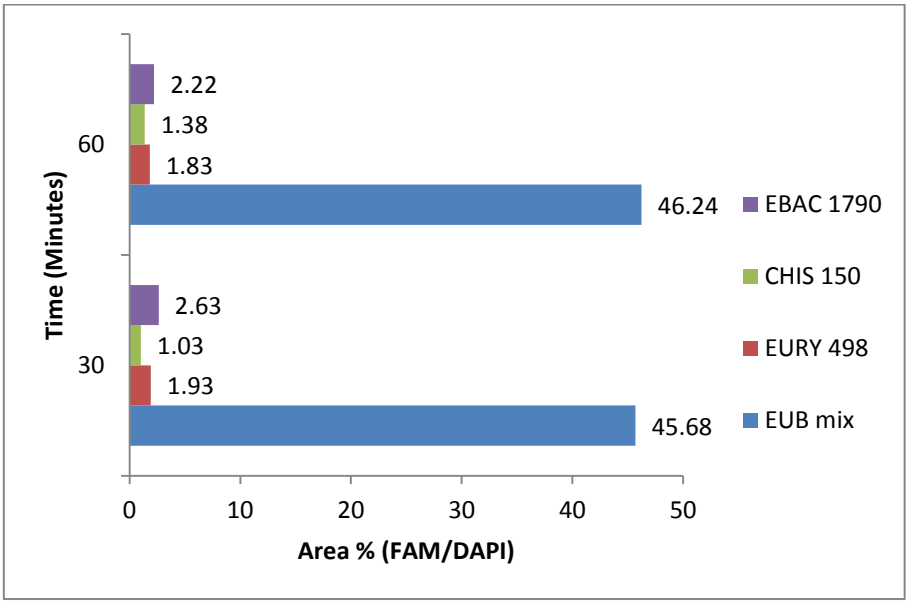


Figure 17. Representation of percentage hybridization of oligonucleotide probes over the total number of DAPI stained cells from the negative control samples.

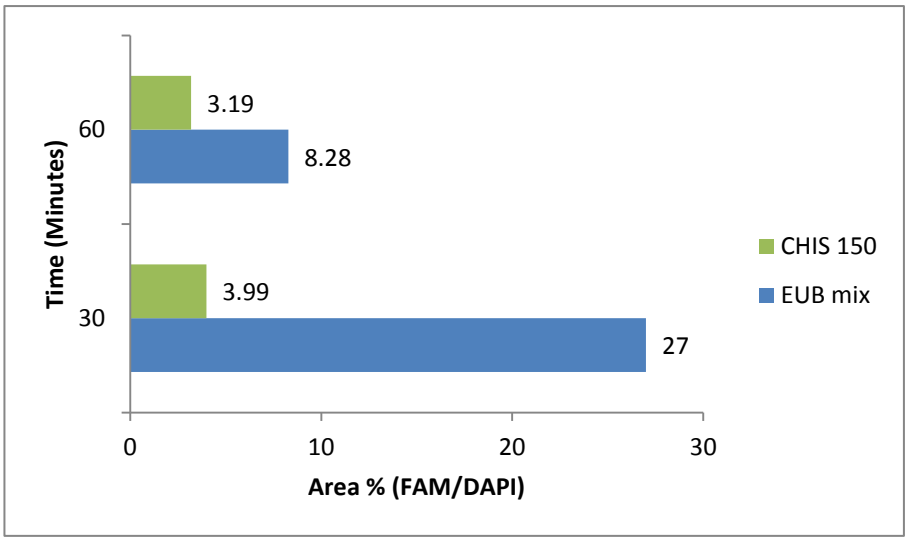


Figure 18. Representation of percentage hybridization of oligonucleotide probes over number of DAPI stained cells after sterilization treatment.

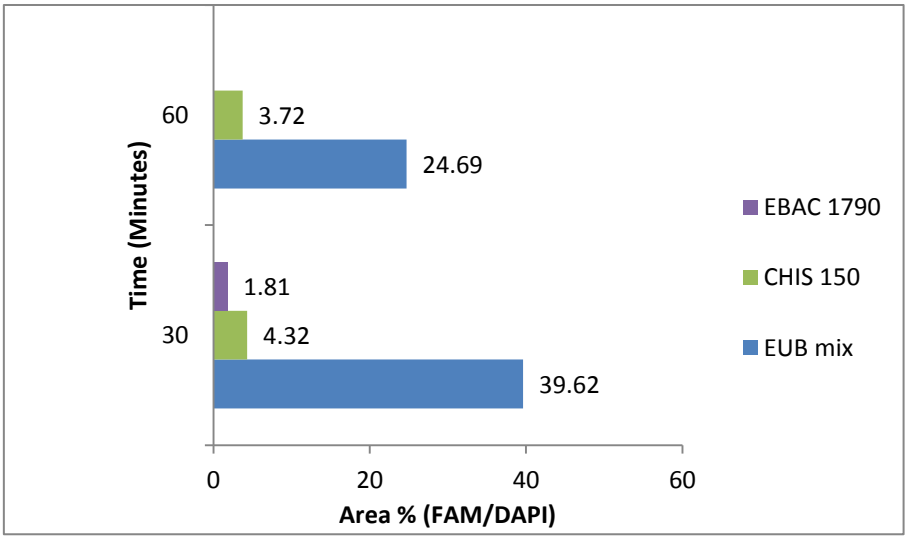


Figure 19. Representation of percentage hybridization of oligonucleotide probes over number of DAPI stained cells after boiling treatment.

The results obtained from our experiment could be compared to other studies conducted achieving similar conclusions (Ren *et al.*, 2008). It was noted that the bacterial population was not majorly affected by boiling and its exposure time. However, boiling treatment was able to eliminate the occurrence of methanogenic bacteria at 30 and 60 minutes. Boiling for 60 minutes however, suppressed or eliminated the population of hydrogen producing species such as Enterobacter from the samples, whereas *Clostridium* remained unaffected even after 60 minutes.

Studies have shown that heating sludge at high temperatures for a longer period can be lethal to hydrogen producing bacteria (Baghchehsaraee *et al.*, 2008, Liu and Wang, 2012). This phenomenon was also reported by Zhu and Berland, suggesting that an increase in temperature and treatment time can also affect hydrogen producing bacterial capabilities thus a lower hydrogen yield can be expected during anaerobic fermentation (Hawkes *et al.*, 2002). The variation of hydrogen production amongst pre-treatment methods could also be attributed to the type of microorganisms present in the seed sludge and its density. Studies (Mu *et al.*, 2007, Chang *et al.*, 2011) also suggested that depending on the source of seed sludge, hydrogen consuming bacteria such as homoacetogens can survive harsh temperatures and hydrogen producing bacteria such *Enterobacter* spp. can be easily destroyed.

Even during heat treatment the *Clostridium* group displayed an increase in hybridization. Spore forming hydrogen generating bacteria such as *Clostridium* are able to form protective spores when under extreme conditions such as high temperatures, but methane producing microorganisms lack this ability (Zhu and Béland, 2006, Wang and Wan, 2008a). Hence, our results show no hybridization for the phylum Euryarchaeota after sterilization and heat treatment because of their inability of forming spores. A study conducted by Luo, demonstrated the significant decrease in COD when heated seed sludge was used as an inoculum. The COD reduced up to 89% and the hydrogen yield increased when compared to untreated sludge. Therefore implying that heat treated sludge enhances COD reduction in wastewater and can be a useful tool in fermentation studies (Luo *et al.*, 2010a).

Similar results were obtained from a study using heat treated anaerobic granular sludge; anaerobic granules were boiled (100°C) for 0.5-4 hours. The effect of boiling showed that heat treatment had a significant impact on the microbial community, resulting in various hydrogen productions. The maximum hydrogen yield produced was 2.14 moles of hydrogen

per mole of glucose using sludge that was treated for a period of four hours. The treated sludge demonstrated the lowest colony forming unit concentration at a pH of 5.5. Thus, this study had concluded that the conditions chosen for heat treatment should be specific to the type of sludge used to select suitable hydrogen producing microorganisms (Alibardi *et al.*, 2012).

Research conducted by (Lay *et al.*, 2011) evaluated hydrogen production rate using heat treated sludge at temperatures 60-97°C for an exposure time of 20-60 minutes. The highest hydrogen production was observed with sludge treated at 70°C for 50 minutes. It was noted that thermally treated sludge displayed a higher conversion of cellulose to hydrogen than that demonstrated by untreated sludge.

4.4 Conclusions

- Hydrogen producing bacteria can be enhanced by thermal pre-treatment of sludge used from municipal wastewater treatment plants.
- Fluorescent in-situ hybridization was effective in identifying the active cells after pre-treatment.
- Changes in microbial communities was successfully identified and quantified by FISH.
- Both thermal pre-treatment methods were effective in eliminating hydrogen consuming bacteria.
- Thermal pre-treatment for 30 minutes of boiling was effective in eliminating methanogens completely from the sample.
- Hydrogen producing *Clostridium* sp. was the most prominent cluster identified.
- Longer exposure period affected the hydrogen producing consortia. Enterobacter population was eliminated after 60 minutes exposure to temperature.

CHAPTER FIVE:
EVALUATION OF CONTINUOUS BIOHYDROGEN PRODUCTION POTENTIAL OF PRE-TREATED SUGARCANE BAGASSE AND SEWAGE SLUDGE IN AN ANAEROBIC DARK FERMENTATION PROCESS AT LABORATORY SCALE

5.1 Introduction

Dark fermentation appears to be the most reliable method using a range of waste sources (wastewater, solid waste and biomass) to produce biohydrogen at a rapid pace. The waste substrates are available in abundant quantities to meet the demand for energy conversion by fermentative hydrogen production (Hallenbeck *et al.*, 2012). Even though this approach is attractive, the major limitation lies with natural metabolic processes. The lack of knowledge on the metabolism of hydrogen producing bacteria makes it difficult to metabolically engineer microorganisms to produce hydrogen (Pandu and Joseph, 2012). Thus, work should be carried out to improve low conversion efficiency of substrates to hydrogen and to enhance activity of hydrogen producing bacteria (Hallenbeck, 2009).

Factors such as substrate type, biohydrogen producing inoculum, pH and temperature influence the metabolic pathways for hydrogen synthesis. (Khanal *et al.*, 2004, Hu *et al.*, 2005). Recent studies have also focused on factors such as the effect of external iron concentration which have shown to diminish hydrogenase activity under depleted iron concentration in the media (Lee *et al.*, 2001). Further studies have also shown the use of nanoparticles to improve the bioactivity of hydrogen producing bacteria (Han *et al.*, 2011a, Zhao *et al.*, 2013). Nanoparticles have an affinity for electrons which enable further reduction of protons to hydrogen resulting in a higher hydrogen production rate (Beckers *et al.*, 2013b). Nanoparticles can be classified by the type of material into metallic, semiconductor and polymeric nanoparticles (Liu, 2006). Using nanoparticles can enhance chemical reaction rates which can increase hydrogen production rate during fermentation (Gadhe and Gupta, 2007).

In this study, we have investigated the effect of external factors such as usage of sugarcane bagasse hydrolyslate, pH, iron and magnetite nanoparticle concentrations on hydrogen production potential. The main objective of this investigation was to conduct a kinetic study

of operational parameters and attempt to enhance biohydrogen production using nanotechnology.

5.2 Materials and methods

5.2.1 Seed microorganisms (Thermal pre-treatment)

The inoculum pre-treatment used for this case was chosen from the optimization studies done in chapter four. The inoculum sludge used in this study was obtained from an anaerobic holding tank situated in a domestic wastewater treatment plant in KwaZulu Natal, South Africa. Prior to use, sludge was heated at 102°C in an oven (Incotherm, economy) for 30 minutes to enrich for hydrogen producing bacteria by inactivating methanogenic and other hydrogen consuming bacteria.

5.2.2 Acid pre-treatment of sugarcane bagasse

The Substrate pre-treatment method chosen was adapted from the optimization study conducted in chapter three. Fresh sugarcane bagasse used in this study was received from Kashmeers sugarcane juice Ltd, Durban South Africa. Samples were sun-dried, blended, milled and sieved to a size $\leq$ 2 mm and stored at room temperature. The lignocellulosic materials were steam pretreated by 3% sodium hydroxide hydrolysis performed for 60 minutes using autoclave at 121°C and 1.4 kg/cm^3.

5.2.3 Preparation of magnetite nanoparticles

Magnetite nanoparticles used for experimentation were prepared by adding 6.2g $FeCl_3$ and 4g $FeCl_2.4H_2O$ in 25ml of deionized water and 1ml of concentrated hydrochloric acid in a 500ml glass beaker. In a second beaker 15g of sodium hydroxide was dissolved in 250ml of deionized water by heating at 80°C. Argon was then purged into the sodium hydroxide solution for 10 minutes followed by vigorous mixing. The solution from the first beaker was

then added dropwise to the second beaker forming a black precipitate. Thereafter, the precipitate was left to settle and washed several times until pH reached 6.8-7.2 (Nasr *et al.*, 2015) .

5.2.4 Experimental procedures

Batch experiments were conducted in 150ml glass bottles containing a food to microorganism ratio of 1.5 (sugarcane bagasse hydrolysate and heat treated sludge). The total working volume in the reaction bottles were fixed at 100ml. A one litre nutrient solution was prepared containing 80g NH_4HCO_3, 1.24g KH_2PO_4, 0.1g $MgSO_4$, 0.01g NaCl, 0.01g $Na_2MoO_4.2H_2O$, 0.01g $CaCl_2.\ 2H_2O$, 0.0015g $MnSO_4.7H_2O$, 0.0278g $FeCl_2$. The batch experiments were carried out using various operational parameters, variation in pH (3, 4, 5, 6, 7 and 9); substrate to biomass ratio (0.5, 1.5, 2.5, 3.5, 4.5 and 5.5); Fe^{2+} concentrations (0, 50, 100, 200, 300 and 400 mg/L) and magnetite nanoparticle concentrations (0, 50, 100, 200, 300 and 400 mg/L). The batch experiments were conducted at 30°C in the dark. Water displacement method was used to collect and measure the biogas produced (Appendix 2, Figure 28).

5.2.5 Analytical methods

Volatile suspended solids (VSS) and chemical oxygen demand were measured following the techniques described in Standard Methods. Carbohydrates were determined the phenol–sulfuric acid method using glucose as a standard. For scanning electron microscopy analysis, the samples were dried, then mounted on the stub by double-sided tape and sputtered with gold. The hydrogen gas composition in the biogas was determined by a gas chromatograph (Agilent 7820) equipped with a thermal conductivity detector. The instrument contained a GS-Gaspro (30 m x 0.32 mm I.D) packed column. Nitrogen was used as the carrier gas with operating temperatures of the oven, detector and injection port were 80, 250, 120°C, respectively. Analysis of volatile fatty acids was also carried out using a gas chromatograph (Shimadzu GC-2014) equipped with a flame ionization detector and a Zebron ZB-FFAP (30m x 0.25mm x 0.25µm) capillary column. The oven temperature was programmed at 100°C for 2 min then increased to 160°C at a ramp rate of 8°C.min^{-1} at a hold time of 0.5 min and nitrogen was also used as the carrier gas.

5.2.6 Calculations and Gompertz model

The modified Gompertz model (Equation 2) was used to fit the cumulative hydrogen production in each batch experiment (Winsor, 1932).

$$H(t) = P \times \exp\left\{-\exp\left(\frac{R_m \times e}{P}(\lambda - t) + 1\right)\right\} \quad (2)$$

Where,

H is the cumulative hydrogen production, P is the hydrogen potential (mL-H_2), R_m is the maximum hydrogen production rate (mL-H_2 h^{-1}), t is the incubation time (h), and λ is the duration of the lag phase (h).

5.2.7 Substrate to biomass (S/X) ratio

Volumes of substrates and seed were calculated based on a substrate-to-biomass (S/X) ratio using the equation (Equation 3).

$$S/X = \frac{V_{substrate} \times COD_{total}}{V_{seed} \times VSS} \quad (3)$$

Where $V_{substrate}$ is the volume of substrate (L); COD_{total} is the total chemical oxygen demand (g/L); V_{seed} is the volume of seed (L); VSS is the volatile suspended solids (g/L) (Nasr *et al.*, 2015).

5.2.8 Microbial analysis

5.2.8.1 DNA Extraction

In this study total genomic DNA was isolated from heat treated sludge samples (anaerobic sludge) taken from each fermentation experiment. DNA was extracted from 0.3g of wet weight biomass using the NaTCA method (Seviour and Nielsen, 2010). Two microliters of sample was centrifuged at 7.000 × *g*, 5 minutes, 4°C. The supernatant was decanted and the pellet was resuspended in 1500µl NaTCA lysis buffer (4.5 M NaTCA, 6 × lysis buffer, 50% (v/v) antifoam and DEPC treated distilled water). Cells were homogenized for 3 minutes with 0.6g of 0.1mm diameter glass beads. The mixture was then pelleted (at 7.000 *g*, 5 minutes, 4°C.) and the supernatant was transferred into a new microcentrifuge tube and mixed with 0.6 vol 2-propanol. The tubes were placed on ice for 15 minutes to precipitate the nucleic acid fraction before being centrifuged at 13.000 × *g*, 5 minutes, 4°C. The supernatant was discarded and the pellet was washed twice with 70% (w/v) ethanol and left to air dry for 15 minutes to remove excess ethanol. The pellet was then resuspended with TE buffer (1 M Tris-HCl (pH 8.0) and 0.5 M EDTA) and incubated at 37°C for 15 minutes. After incubation the pellet was mixed and centrifuged (13.000 × *g*, 30 seconds, 25°C) to transfer the supernatant to a new tube and stored at -20°C till further use.

5.2.8.2 Spectrophotometric analysis of DNA

The purity and concentration of genomic DNA was determined by Nanodrop (ND-1000) Spectrophotometer. One microliter of genomic DNA was measured using absorbance ratios 260/230 and 260/280nm.

5.2.8.3 The Polymerase chain reaction

Polymerase chain reaction conditions were optimized for *Bacillus* spp., *Clostridium* spp., Hydrogenase gene, *Enterobacteriaceae*, bacterial and Universal primers listed in Table 4. Template DNA concentration of 10ng/ml DNA was prepared and used for PCR. The PCR

mixture used was a 25µl reaction volume containing 17.38µl deionized water, 1.87µl Taq buffer, 0.5µl dNTPs (10Mm), 0.25µl Taq polymerase (5 U/ml), 1µl of each primer (5mM) and 1ul of template DNA. Veriti TM 96-well Thermal Cycler (Applied Biosystems) was used to amplify DNA by an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds. Primer annealing at 53°C for 1 minute for bacteria, at 55°C for 1 minute for Universal and *Bacillus* spp., at 56°C for 1 minute for Enterobacteriaceae, at 58°C for 1 minute for *Clostridium* spp. and hydrogenase gene. Elongation was performed at 72°C for 30 seconds and a final extension at 72°C for 4 minutes.

Agarose gel electrophoresis was used to visualize amplified PCR products on 1.2% (w/v) agarose gel stained with ethidium bromide. 100bp and 1kb gene rulers were used to approximate the size of amplicons produced. Gel imaging was completed using Vacutec gel documentation system.

5.2.8.4 Quantitative polymerase chain reaction (QPCR)

Real time PCR was carried out using primers, *Clostridium* spp. (16S-f &16S-r), *Enterobacteriaceae* (f&r) and Hydrogenase gene (HGf & HGr). The quantification and expression of primers in each sample were analysed using a real–time PCR machine (C-1000 Touch, CFX 96, Bio-Rad Laboratories Pty Ltd, USA). The reactions were performed using 4µl of the Sso fast Eva green Master Mix, 0.4µl of each primer (final concentration, 5µM), 2µl of template DNA and nuclease free water was added to a final volume of 10µl.

The amplification of target DNA was performed according to a modified protocol (De Sá *et al.*, 2011) as follows:

Clostridium spp. (16s-f &16s-r) and *Enterobacteriaceae* primers: initial hold of 3.30 minutes at 95°C, followed by 40 cycles consisting of 30 seconds each of denaturation at 95°C, annealing at 58°C and 62°C respectively with extension at 72°C. Hydrogenase gene primer (HGf & HGr) cycle conditions began with initial hold of 10 min at 95°C, followed by 40 cycles consisting of denaturation at 95°C for 15 seconds, annealing at 58°C for 1 minute and

extension at 72°C for 30 seconds. A final stage was added to the real-time cycle for melting curve analysis, the temperature was increased at 0.5°C every 10 s from 55 to 95°C.
During each cycle, negative controls containing no genomic DNA were included to verify any possibilities of contamination.

Quantification of 16S rDNA concentration within the samples was calculated by comparing the Cq values of the sample with the corresponding standard curves. Equation 4 was used to calculate the copy numbers of targeted 16S rDNA in each sample.

In the equation Avogadro's constant (6.02×10^{23}) was used to assume the base pairing of double stranded DNA and 660 for the average molecular weight (Tan *et al.*, 2013).

$$16S\ rDNA = \frac{16S\ rDNA\ concentration\ (g/ml) \times 6.02 \times 10^{23}\ (copy/mole)}{16S\ rDNA\ amplicon\ size\ (bp) \times 660\ (g16S\ rDNA\ mol/bp)} \quad (4)$$

Table 4. Sequences of primers used for conventional and quantitative polymerase chain reaction

Primer name and targeted organism	**Sequence (5 ´- 3´)**		**Product Size (bp)**	**References**
	Forward primer	**Reverse primer**		
Bacillus spp. (Pf & Pr)	GAG TTA GAG AAC GGT ATT TAT GCT GC	CTA CTG CCG CTC CAT GAA TCC	199	(Schraft and Griffiths, 1995)
Clostridium spp.(16S-f &16S-r)	AGC GTT GTC CGG ATT TAC TG	TTC GCC ACT GGT ATT CTT CC	182	(De Sá *et al.*, 2011)
Hydrogenase gene (HGf & HGr)	AAG AAG CTT TAG AAG ATC CTA A	GGA CAA CAT GAG GTA AAC ATT G	259	(De Sá *et al.*, 2011)
Enterobacteriaceae	CAG GTC GTC ACG GTA ACA AG	GTG GTT CAG TTT CAG CAT GTA C	512	(Fazzeli *et al.*, 2012)
Universal (27f & 1492r)	AGA GTT TGA TCM TGG CTC AG	GGY TAC CTT GTT ACG ACT T	1500	(Turner *et al.*, 1999)
Bacteria (P338 & P518	ACT CCT ACG GGA GGC AGC AG	ATT ACC GCG GCT GCT GG	236	(Boon *et al.*, 2002)

5.3 Results and discussion

5.3.1 Effect of pH on hydrogen fermentation

Individual batch tests were observed until the hydrogen production from each bottle ceased within 12 h. This short fermentation time could be attributed to a high degradability of SCB hydrolysate. Additionally, the hydrogen cumulative profile witnessed a short lag phase (mostly less than 1 h), which was mainly due to acclimatization of hydrogen-producing bacteria in anaerobic fermenters operated for long periods before starting batch experiments. Biogas analysis indicated that the anaerobic fermentation produced mainly H_2 and CO_2, suggesting that no methanogens bacteria were active in the experiments. This was mainly due to subjecting the mixed culture to thermal pre-treatment at 102°C for 30 min (Nasr *et al.*, 2013).

Table 5. Effect of pH on hydrogen fermentation and COD mass balance from SCB hydrolysate

pH	R_m (mL/h)	P_m (mL)	SHPR (mmol- /g-VSS.d)	HY (mol/mol-glucose)	COD removal (%)	Biomass yield (g/g-$COD_{converted}$)	COD Balance* (%)	HAc/HBu
3	2.0	12.9	9.7	0.4	28.8	0.33	83.6	0.86
4	3.4	22.3	16.1	0.7	35.4	0.36	82.6	0.98
5	4.8	28.4	22.4	0.8	39.5	0.36	81.4	1.11
6	3.7	26.0	17.2	0.8	38.8	0.37	81.6	1.08
7	3.7	24.3	16.1	0.7	45.0	0.48	82.2	0.62
9	2.9	19.1	12.1	0.6	47.8	0.51	81.2	0.63

*Based on 1.42 g-COD/g-VSS; 8 g-COD/g-H_2

The results of hydrogen production from SCB hydrolysate at different initial pH are listed in Table 5. Results showed that the coefficient of determination (r^2) was 0.80 – 0.85 for all Gompertz data. The hydrogen production improved from 12.9 to 28.4 mL-H_2, when the initial pH from 3 to 5 (*r* 0.993, *p* 0.078). A further increase in pH resulted in a drastic decrease in hydrogen production reaching 19.1 mL-H_2 at the initial pH of 9 (*r* -0.997, *p* 0.003). Similarly, the specific hydrogen production rate (SHPR) was elevated from 9.7 to 22.4 mmol-H_2/g-VSS.d when the initial pH was increased from 3 to 5 (*r* 0.999, *p* 0.003). Subsequently, the SHPR dropped to 12.1 mmol-H_2/g-VSS.d when the initial pH was raised to 9 (*r* -0.959, *p* 0.041). The hydrogen yield (HY) increased from 0.38 to 0.83 mol-H_2/mol-glucose on varying the initial pH from 3 to 5 (*r* 0.993, *p* 0.073), and then continually declined to 0.56 mol-H_2/mol-glucose at the initial pH of 9 (*r* -0.998, *p* 0.002).

Our results indicate that hydrogen production from SCB was strongly pH-dependent, and the performance of hydrogenase system could be enhanced at the optimum initial pH (5). Additionally, lowest hydrogen production was observed at the initial pH values of 3 and 9, indicating that extremely acidic or alkaline conditions are unfavorable for fermentative hydrogen production. The major advantage of weak acidic optium conditions for hydrogen production is the inhibition of methanogenic activity at pH 5 to 6 in anaerobic processes, thus indirectly stimulating hydrogen producers within the system (Nasr *et al.*, 2015). However, at highly acidic conditions (pH 3) hydrogen production was dramatically reduced due to biomass inhibition. Similarly, (Duangmanee *et al.*, 2007) observed that a low fermentation pH of approximately 4 inhibits hydrogen production. Additionally, Zhang et al. (2007) reported that a high pH inhibited the growth of microbes, and their abilities to produce hydrogen. Our results are in agreement with (Lay, 2000), who reported that at pH higher or lower than 5, the hydrogen production decreased, and had terminated below pH 4. Moreover, some studies noted that a pH of 5.5 – 6.0 is an ideal range for biohydrogen production (Nandi and Sengupta, 1998).

Table 5 shows the volatile fatty acids (VFA) at various initial pH levels. Most of the VFAs were analysed as acetic acid (HAc) and butyric acid (HBu), whereas the propionic acid (HPr) was below the analytical limit. Formation of HAc and HBu are considered optimum for effective hydrogen production. It was found that the maximum HY was produced together with the molar ratio of acetate-butyrate (HAc/HBu) equals to 1.11 at the initial pH of 5. A higher HAc/HBu ratio is theoretically favorable for obtaining higher HY, since complete

conversion of 1 mol-glucose into acetate yields 4 mol-H_2 (Reaction 7), while only 2 mol-H_2 is formed when butyrate is the end product (Reaction 8). In practice, a theoretical yield of 2.6 mol-H_2/mol-hexose is achieved when the catabolism is driven through a mixed acid fermentation according to Reaction 9. After the incubation period, accumulation of VFAs resulted in decreasing the system pH, leading to process inhibition (Singh *et al.*, 2013).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (7)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (8)$$

$$C_6H_{12}O_6 \rightarrow 0.6\ CH_3COOH + 0.7\ CH_3CH_2CH_2COOH + 2CO_2 + 2.6\ H_2 \quad (9)$$

COD mass balance of the fermenters was calculated considering all the metabolites products releases, the hydrogen gas generated, and the equivalent COD for the biomass produced. The COD mass balance was within the range of 81 – 84%, indicating that fraction of the total COD was stored for cell maintenance. The highest COD reduction efficiency achieved was 45.0 – 47.8% at pH of 7 – 9; however, the HY was lower than the optimum level. Additionally, the biomass yield recorded at pH 7 – 9 (i.e. 0.48 – 0.51 g-COD/g-$COD_{converted}$), was higher than pH 3 – 6 (0.33 – 0.37 g-COD/g-$COD_{converted}$). This indicates that pH of 7 – 9 is ideal for the utilization of COD as an energy source for biomass synthesis and growth other than hydrogen production. These results were compared to the typical biomass yield of 0.15 g-COD/g-$COD_{converted}$ that was reported for hydrogen producers (Chen *et al.*, 2001) and 0.4 g-COD/g-$COD_{converted}$ for biological hydrogen production from a continuously stirred reactor (Hafez *et al.*, 2010).

Since 1 mol of glucose can theoretically produce a maximum of 4 mol of H_2 considering acetic acid as the final product of the reaction (Equation 5), a 20.8% conversion efficiency (related to the maximum theoretical) was achieved at pH 5. These result were comparable to previous reports by (Logan *et al.*, 2002) and (Oh *et al.*, 2003), where glucose conversion efficiencies were 23% at a pH of 6.0 and between 24.2-18.5% at a pH range of 6.2-7.5.

5.3.2 Effect of substrate-to-biomass (S/X) ratio on hydrogen fermentation

The effect of S/X ratio on the performance of hydrogenase system treating SCB hydrolysate was studied. An increase in hydrogen production from 12.2 to 37.5 mL-H_2 was observed with an increase in S/X from 0.5 to 3.5 g-COD/g-VSS (*r* 0.990, *p* 0.010) (Table 6). However, the hydrogen production linearly dropped to 19.4 mL-H_2 when the S/X was lifted to 5.5 g-COD/g-VSS (*r* -0.999, *p* 0.033). Similarly, the SHPR has increased by 63.4% and reached up to 19.4 mmol-H_2/g-VSS.d when the S/X was raised to 3.5 (*r* 0.894, *p* 0.106), after that, a sharp decline in the SHPR was observed owing to a further increasing S/X to 5.5 (*r* -0.999, *p* 0.024). Thus, the maximum cumulative volume of hydrogen and SHPR were achieved at S/X not exceeding 3.5 g-COD/g-VSS.

Table 6. Effect of S/X on hydrogen fermentation and COD mass balance from SCB hydrolysate

S/X	R_m (mL/h)	P_m (mL)	SHPR (mmol-/g-VSS.d)	HY (mol/mol-glucose)	COD removal (%)	Biomass yield (g/g-$COD_{converted}$)	COD Balance* (%)	HAc/HBu
0.5	2.2	12.2	11.5	0.8	21.1	0.33	92.7	1.24
1.5	3.6	23.6	17.4	0.7	28.2	0.33	86.9	1.20
2.5	4.2	31.3	17.3	0.5	39.8	0.42	81.4	1.16
3.5	5.3	37.5	19.4	0.5	40.9	0.45	81.5	0.70
4.5	4.1	26.2	13.6	0.2	32.2	0.50	85.6	0.64
5.5	3.1	19.4	7.0	0.1	39.2	0.50	81.3	0.56

*Based on 1.42 g-COD/g-VSS; 8 g-COD/g-H_2

Our results indicated that operation of the hydrogen fermenter treating SCB hydrolysate using S/X of 3.5 was adequate to produce hydrogen gas. An increase in S/X value above 3.5 would undoubtedly lead to a decrease in the fermentative hydrogen production. This might be due to several factors: 1) Accumulation of soluble metabolites/products at higher S/X ratios, resulted in acidification of bacterial cultures and the inhibition of fermentation process; 2) At higher S/X, microbes could convert only the soluble part of substrate to hydrogen due to rate limiting steps in fermentation process such as hydrolysis or 3) The increase in biomass yield at relatively high S/X ratios, attribute to proliferation of microorganisms other than hydrogen producers (Hafez *et al.,* 2010). Thus, to avoid these inhibition reasons, the S/X ratio in the liquid-phase should be maintained at an optimal value. Our results indicate that lower or much higher S/X ratio than 3.5 were not favorable to increase hydrogen production rate (Li and Ren, 1998, Mullai *et al.,* 2013).

The distribution of metabolites formed during hydrogen fermentation is often a crucial signal in assessing the efficiency of hydrogen production. As listed in Table 6, HAc and HBu were the major aqueous products and their amounts were varying with the change of S/X ratio. It was observed that the HY had a positive agreement with the HAc/HBu molar ratio, where it declined from 0.84 to 0.09 mol-H_2/mol-glucose with a decrease in HAc/HBu from 1.24 to 0.56, respectively (r 0.920, p 0.009). This might be due to the fact that the theoretical HY from glucose with acetate formation of 4 mol-H_2/mol-glucose is twice that of butyrate formation. Thus, the molar ratio of HAc/HBu ratio could be an indicator for evaluating the effectiveness of hydrogen yield.

COD balance at 81.3 – 92.7% validates the reliability of the measured data. The observed biomass yield increased from 0.33 to 0.50 g-COD/g-$COD_{converted}$ with an increase in S/X from 0.5 to 5.5 (r 0.961, p 0.002). The inverse relationship between the biomass and hydrogen yields (r -0.963, p 0.002) demonstrated that the higher biomass yield could enrich the growth of competitors other than hydrogen producers. Similar observations were noted by (Chen *et al.,* 2001) who found that at high substrate concentrations (S/X ratios), a microbial shift occurs leading to an increase in the biomass yield, which was not related to hydrogen producers. Additionally, a low pH value between 3.7 – 4.0 and an increased VSS concentration is an indication of organic acid production and microbial growth.

5.3.3 Effect of Fe^{2+} concentration on hydrogen production

The effects of different Fe^{2+} concentrations (0, 50, 100, 200, 300 and 400 mg/L) on hydrogen fermentation are listed in Table 7. The cumulative hydrogen production was observed to increase from 23.8 mL-H_2 to 37.3 mL-H_2 when Fe^{2+} concentration was increased from 0-200 mg/L (*r* 0.983, *p* 0.017). The HY noted a similar trend, where it lifted from 0.70 to 1.09 mol-H_2/mol-glucose (increased by 57%) with an increase in Fe^{2+} concentration from 0 to 200 mg/L, respectively (*r* 0.979, *p* 0.021). However, increasing the Fe^{2+} concentration to 400 mg/L resulted in a decrease in the cumulative hydrogen production to 14.6 mL-H_2 (*r* -0.994, *p* 0.069), and HY to 0.43 mol-H_2/mol-glucose (*r* -0.995, *p* 0.067). Similarly, the SHPR increased with Fe^{2+} concentration up to 200 mg/L (*r* 0.917, *p* 0.083), and then declined with further increase in Fe^{2+} to 400 mg/L (*r* -0.980, *p* 0.126).

Table 7. Effect of iron concentration on hydrogen fermentation and COD mass balance from SCB hydrolysate

Fe^{2+} (mg/L)	**R_m (mL/h)**	**P_m (mL)**	**SHPR (mmol-/g-VSS.d)**	**HY (mol/mol-glucose)**	**COD removal (%)**	**Biomass yield (g/g-$COD_{converted}$)**	**COD Balance* (%)**	**HAc/HBu**
0	3.8	23.8	18.5	0.7	32.0	0.32	83.8	1.05
50	3.9	25.8	18.6	0.8	33.2	0.34	84.1	1.14
100	4.9	32.0	22.9	0.9	33.3	0.37	86.5	1.18
200	5.2	37.3	24.1	1.1	34.0	0.38	87.9	1.23
300	4.4	28.1	21.3	0.8	32.5	0.30	83.8	1.20
400	3.1	14.6	15.5	0.4	26.2	0.27	84.4	1.03

*Based on 1.42 g-COD/g-VSS; 8 g-COD/g-H_2

The cumulative hydrogen gas produced was observed to be in a linear agreement with Fe^{2+} concentration in the range of 0 – 200 mg/L. However, when Fe^{2+} concentrations were exceeding 200 mg/L, hydrogen production decreased, demonstrating that those concentrations were not favorable for hydrogen production. Thus, much lower or much higher Fe^{2+} concentrations than 200 mg/L were not preferable for hydrogen production from SCB hydrolysate.

Addition of external iron could improve biohydrogen production since hydrogenase, is a key enzyme on hydrogen production and comprises of iron at the active site. Previous studies have indicated that the addition of optimum levels of Fe^{2+} to the fermentation medium could enhance hydrogen production by influencing the hydrogenase enzyme activity (Zhang *et al.*, 2005). Furthermore, hydrogenase present in anaerobic bacteria oxidizes reduced ferredoxin, which is also influenced by Fe^{2+} concentration, to produce molecular hydrogen (Adams *et al.*, 1980). However, an increased iron concentration could be toxic to hydrogen-producing microorganisms (Yang and Shen, 2006). It has been reported that when Fe^{2+} concentration is beyond a threshold range it would decrease the hydrogen production rate due to an increased lag-phase and formation of soluble microbial products (Lee *et al.*, 2001).

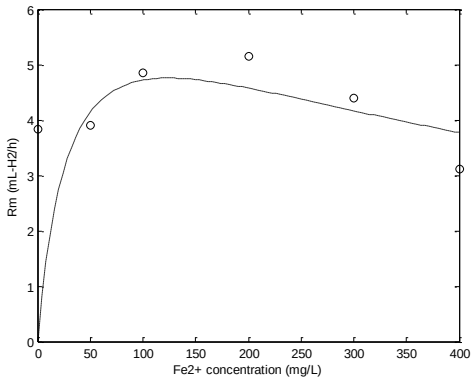
According to previous reports, optimal initial iron concentration was still uncertain for optimum hydrogen yield. (Zhang and Shen, 2006) found that the maximum hydrogen production of 356, 371 and 351mL were obtained at the iron concentration of 800, 200 and 25 mg-$FeSO_4$/L, for 25, 35, and 40ºC, respectively. Moreover, (Zhang *et al.*, 2005) noted that the maximum amount of hydrogen production (2.73 mol-H_2/mol-sucrose) was obtained when the media was supplemented with an higher iron concentration of 1600 mg-$FeSO_4$/L. Furthermore, (Wang and Wan, 2008b) observed a maximum cumulative hydrogen production of 302 mL and a maximum HY of 311 mL/g-glucose at an Fe^{2+} concentration of 300 and 350 mg/L, respectivel. Additionally, (Mohanraj *et al.*, 2014) found a maximum HY of 1.7 mol-H_2/mol-glucose and 5.19 mol-H_2/mol-sucrose with 25 mg/L of ferrous iron supplementation. Possible reasons for differences may be dissimilarity in substrate concentration, source of microbes and/or dominant species, initial pH and/or operated temperature.

Andrew's inhibition model (Equation 5) was applied to study the inhibitory effect of Fe^{2+} concentration on the hydrogen production.

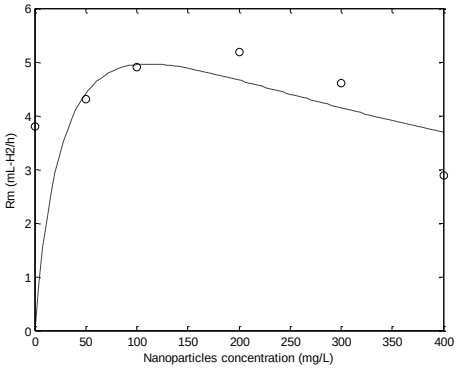
$$R = (R_m \times S)/(K_S + S + S^2/K_i) \quad (5)$$

Where R_m is the maximum rate of hydrogen production (mL/hr); K_s is the saturation constant (mg/L); K_i is the inhibition constant (mg/L); and S is the concentration of Fe^{2+} in mg/L.

Through a non-linear regression between hydrogen production rate and Fe^{2+} concentrations, the values of K_s and K_i were estimated as 32 and 482 mg/L, respectively. A high correlation coefficient of 0.87 (Figure 20a) suggests the suitability of Andrew's model in describing the inhibitory effect of Fe^{2+} concentration on the hydrogen production.



a)



b)

Figure 20. Kinetic analysis of hydrogen production rate at different concentrations of: a) Iron b) Magnetite nanoparticles

The main soluble metabolites produced by the mixed cultures in this study were HAc and HBu, which are typical for hydrogen production. The higher HAc to HBu molar ratio in the fermentation products would translate to higher HYs. A COD mass balance of 84 – 88% verifies our data reliability. When the Fe^{2+} concentration was lower than 200 mg/L, the COD degradation efficiency was between 32 and 34%, however, the COD degradation efficiency had the trend to decrease with an increase in Fe^{2+} concentrations from 200 to 400 mg/L.

Additionally, the highest biomass yield of 0.37 – 0.38 g-COD/g-$COD_{converted}$ was observed when the Fe^{2+} concentration was between 100 and 200 mg/L. Thus, we could also conclude that the biomass yield followed the same trend with the HY, indicating that the concentrations of Fe^{2+} affected both the cultivation and the hydrogen-producing activity of the bacteria. These results indicated that the bioactivity of hydrogen-producing bacteria used was promoted by the addition of a certain Fe^{2+} concentration. The final pH value had the trend to increase with increasing Fe^{2+} concentrations.

5.3.4 Effect of magnetite nanoparticles on hydrogen fermentation

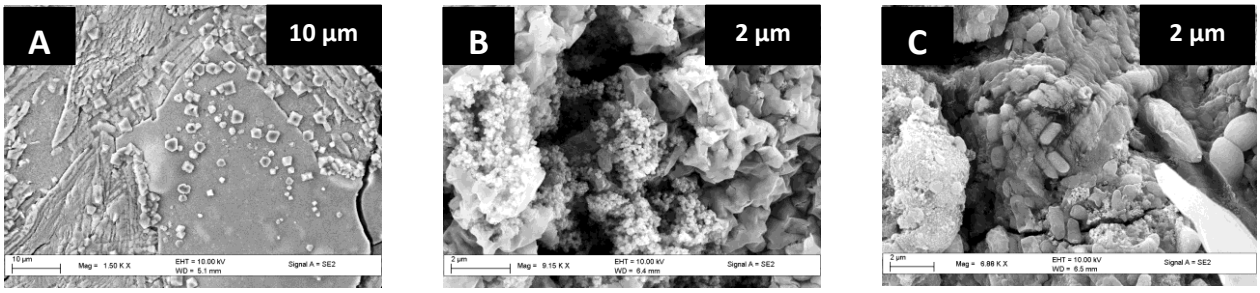


Figure 21. Scanning electron microscopy of: a) Magnetite nanoparticles; b) Iron addition (200mg/L); c) Sludge immobilized on magnetite nanoparticles (200mg/L)

Magnetite nanoparticles of various concentrations were tested for their efficacy on enhancing hydrogen production during batch fermentation. SEM analysis of the fermentation broth has shown the presence of mixed culture of bacteria on the surface of nanoparticles (Figure 21) Average diameter of the nanoparticles used for the study was approximately 50 ± 5 nm.

As listed in Table 8, the concentration of magnetite nanoparticles increased from 0 to 400 mg/L during fermentation of SCB hydrolysate. The volumetric hydrogen production was significantly increased from 24.2 mL-H_2 at 0 mg/L to 40.2 mL-H_2 at 200 mg/L, when the magnetic nanoparticle concentration in the media was increased from 0 mg/L (control) to 200mg/L (r 0.967, p 0.033). However, further increase in magnetite nanoparticle concentration above 200 mg/L adversely affected the hydrogen production efficiency. This

was evident where the cumulative hydrogen had dropped to 20.4 mL-H_2 when magnetite nanoparticle concentration was increased to 400 mg/L (*r* -0.991, *p* 0.085). Similarly, the HY elevated from 0.71 to 1.18 mol-H_2/mol-glucose when nanoparticles concentration increased from 0 to 200 mg/L (*r* 0.966, *p* 0.035), after which it declined to 0.60 mol-H_2/mol-glucose at 400 mg/L (*r* -0.990, *p* 0.088).

Table 8. Effect of magnetite nanoparticles concentration on hydrogen fermentation and COD mass balance from SCB hydrolysate

Magnetite nanoparticles (mg/L)	**R_m (mL/h)**	**P_m (mL)**	**SHPR (mmol- /g-VSS.d)**	**HY (mol/mol-glucose)**	**COD removal (%)**	**Biomass yield (g/g-$COD_{converted}$)**	**COD Balance* (%)**	**HAc/HBu**
0	3.8	24.2	18.6	0.7	28.0	0.31	86.3	0.94
50	4.3	29.0	20.7	0.8	29.7	0.34	87.2	1.16
100	4.9	36.0	23.1	1.1	33.9	0.36	86.9	1.36
200	5.2	40.2	24.2	1.2	35.6	0.37	87.2	1.48
300	4.6	32.6	22.1	1.0	29.6	0.35	88.6	1.23
400	2.9	20.4	14.2	0.6	25.9	0.29	86.5	1.09

*Based on 1.42 g-COD/g-VSS; 8 g-COD/g-H_2

Nanoparticles enhance the fermentation reaction due to their high surface area which will allow for a better microbial activity, as well as their ability to provide unique physical and chemical properties (Mohanraj and Chen, 2006). Zhang and Shen (Zhang and Shen, 2007a) suggested that nanoparticles may encourage hydrogen production by surface effect and quantum size effect. However, the higher concentration of metal nanoparticles might have caused toxicity and produced reactive oxygen species that may have affected the growth of hydrogen producing microorganisms (Simko *et al.*, 2011).

In this study, the maximum hydrogen production rate was 5.2 mL/hr, which is much lower than the reported value of 10.4 mL/hr for the hematite nanoparticles concentration of 200 mg/L (Hongliang *et al.*, 2011). Additionally, our study achieved a maximum HY of 5.9 mmol/g-COD at 200 mg/L, which is comparable to that observed by (Zhao *et al.*, 2011) of 7.97 mmol/g-COD at an optimum mesoporous Fe_3O_4 nanoparticles of 400 mg/L. Additionally, (Han *et al.*, 2011b) found that the optimum hematite nanoparticles concentration was 200 mg/L, which recovered a HY of 9.34 mmol-H_2/g COD. Furthermore, (Mohanraj *et al.*, 2014) noted the maximum HYs of 10.78 and 14.00 mmol/g COD, at iron oxide nanoparticles concentration of 125 and 200 mg/L, respectively. Another report by (Beckers *et al.*, 2013b) highlights the effects of Fe NP encapsulated in porous silica on biohydrogen production, and observed a HY of 11.46 mmol/g-COD at a concentration of 135 mg/L using glucose as a substrate. On the other hand, (Mullai *et al.*, 2013) studied an effect of nickel oxide NP on biohydrogen production from glucose, and observed a HY of 13.53 mmol/g-COD at concentration of 5.67 mg/L. Research by (Malik *et al.*, 2014) assessed the effect of iron oxide NP on biohydrogen generation from distillery wastewater and detected a HY of 1.97 mmol/g-COD at concentration of 50 mg/L. The above dissimilarities in the effect of nanoparticles on H_2 yield could be as a result of the type of nanoparticles, selection and concentration of substrates, medium pH and source of seed sludge.

The distribution of metabolites formed through fermentative hydrogen production is often an important signal in evaluating the efficiency of hydrogen producing microorganisms. As shown in Table 8, HAc and HBu were the major components in liquid metabolites produced during the fermentation, but HPr was produced in low quantities. The HAc/HBu molar ratio increased sharply to the control. This had a positive effect on hydrogen yield. Similar findings were reported by Nasr et al. (Nasr *et al.*, 2015) where the fermenter inoculated with sludge immobilized on maghemite nanoparticles showed a higher HAc/HBu ratio of 0.72 as compared to control (HAc/HBU= 0.63).

Addition of nanoparticles at a concentration of 200 mg/L attained a better COD removal efficiency of 35.6% and cell yield of 0.37 g-COD/g-$COD_{converted}$. Optimum nanoparticles concentration enhanced the microbial (hydrogenase) activity by providing more surface area for biohydrogen (Mullai *et al.*, 2013). However, concentrations higher than 200 mg/L impacted negatively on hydrogen production from hydrolysate of SCB. This could be due to

the toxicity effect of nanoparticles at high concentrations which could have resulted in cell wall penetration, cell wall breakage and oxidative stress (Gadhe *et al.*, 2015).

From the above results, it can be observed that the biohydrogen produced at different magnetite nanoparticles concentrations were slightly higher than those at similar Fe^{2+} concentrations. Hence, it is believed that the enhancement effect of magnetite nanoparticles on biohydrogen production from SCB hydrolysate could be mainly attributed to the iron content in nanoparticles. Iron is very important in hydrogen production and it is a fundamental component of ferredoxin. The electron carrier ferredoxin in hydrogenase plays an important role in the fermentative hydrogen production.

The biochemical process either provides the hydrogenases with reducing power from hydrogen oxidation or act as "electron sinks" (Reaction 7) (Zhang and Shen, 2007a). This reaction is catalysed by a dimetallic iron only [FeFe]-hydrogenase in *Clostridia*, which obtains protons by the reduced form either of ferredoxin or of NADH (Vignais and Billoud, 2007). The role of Fe-hydrogenase in biological hydrogen production was intensively discussed in a review study by Das et al. (Das *et al.*, 2006).

$$H_2 \leftrightarrow 2H^+ + 2e^- \qquad (7)$$

However, the surface effect of nanoparticles on enhancing the hydrogen-producing bacteria could be diminished. This might be due to the fact that, the wider surface area could only convey a higher amount of adsorb electrons, but has no influence with the interaction between the adsorbed material and the particle interface (Chen *et al.*, 1996). These observations were in agreement with a previous study by (Zhang and Shen, 2007a) who studied the enhancement effect of gold nanoparticles on biohydrogen production from artificial wastewater. Their study found that the most possible mechanism of nanoparticles on biohydrogen production was quantum size effect other than the surface effect, which was associated to the electron transfer between nanoparticles and protein molecules.

5.3.4 Microbial community analysis

5.3.4.1 Polymerase chain reaction and Quantitative polymerase chain reaction

Bacterial species such as *Enterobacter*, *Clostridium* spp. and *Bacillus* are often reported as the most dominant microbes in H_2 producing reactors (Akutsu *et al.*, 2009, Patel *et al.*, 2014, Nakashimada *et al.*, 2002). Though the metabolic pathways are not well explored, the performance of these microorganisms in H_2 production is directly linked to the hydrogenase gene (Chang *et al.*, 2008). In this study, PCR was used to preliminary identify the known hydrogen producing bacteria in the reactor using 16S rDNA species specific (*Bacillus*, *Enterobacter* and *Clostridium*) and functional gene (hydrogenase gene, hydA)) specific primers (Table 4). The PCR was optimized for each set of primers (Figure 22 and 23). The heat pre-treated samples showed the positive amplification consistently for all the three species of hydrogen producing bacteria and the functional hydrogenase gene (hydA) indicating their presence in heat pre-treated samples.

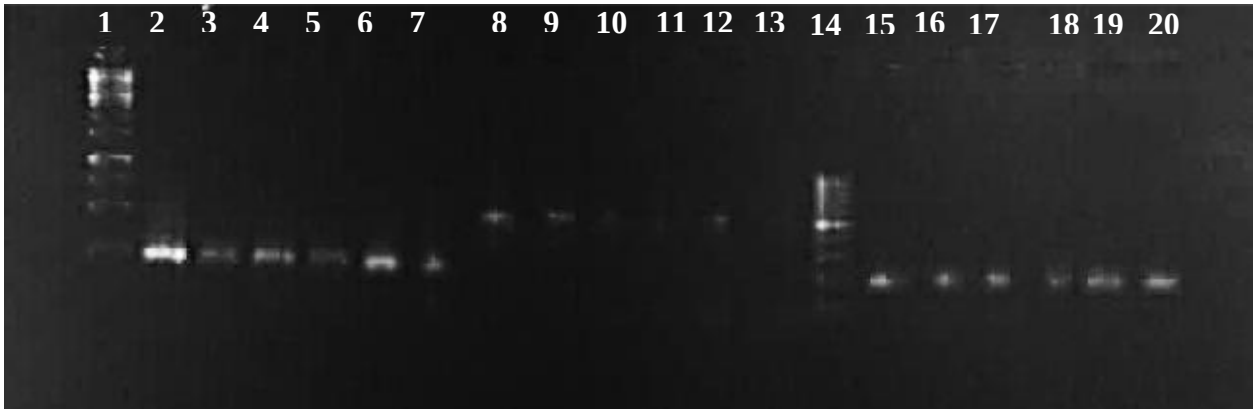


Figure 22. Agarose gel representing PCR products for 1Kb base pair ladder in lane one, lanes 2-7 depicts amplification for bacterial primer (P338 & P518), lanes 8-19 showing Enterobacteriaceae, lane 14 indicates 100bp ladder and 15-20 shows *Clostridium* spp.

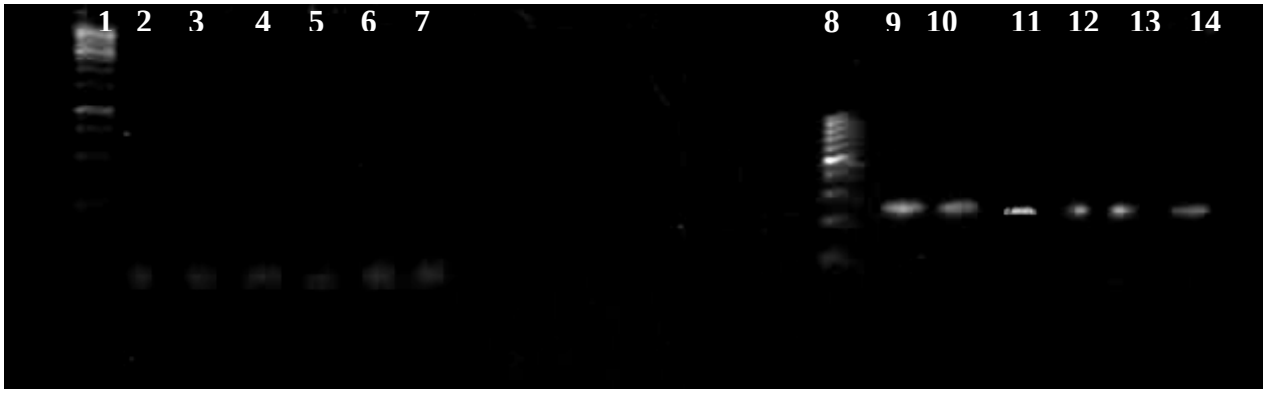


Figure 23. Agarose gel representingPCR products for 1Kb base pair ladder in lane one, lanes 2-7 depicts amplification for Bacillus spp. (Pf & Pr), lane 8 indicates 100bp ladder and lanes 9-14 shows hydrogenase gene.

The extracted DNA from raw sludge (S1), the samples that produced the highest moles of hydrogen from iron (S2) and magnetite optimization experiments (S3) were further subjected to quantitative PCR (QPCR). This was experiment was carried out to understand the correlation of abundance of hydrogen producing bacteria and hydrogen production potential of the reactor based on the hydrogenase gene copy number per gram of total genomic DNA. The standard curve was constructed by plotting the real-time threshold cycle against the copy number of targeted DNA. Purified specific PCR amplicons were used to obtain a standard curve for each QPCR run. The standard curves were obtained from a series of 10-fold dilution, the parameters for the standard curves of amplicons for hydA primer, *Clostridium* spp. and Enterobacteriaceae are displayed in Table 9 below and in Appendix 2.

Table 9. Parameters of QPCR standard curves for primer sets used in this study

Parameter	**Primer**		
	***Clostridium* spp.**	***Enterobacteriaceae***	**Hydrogenase**
Linear range (copies/ng DNA)	$4.84 \times (10^2$ to $10^9)$	$2.97 \times (10^1$ to $10^8)$	$2.2 \times (10^2$ to $10^9)$
R^2 of slope	0.998	0.988	0.999
Slope (standard deviation)	-3.524	-3.499	-3.598
Intercept	39.938	38.095	40.698

Table 9 presents the statistical analysis from the standard curves constructed for each primer set. According to the results obtained, the standard curves for all primers had a 95% confidence interval with no significant slope difference. The accuracy of the QPCR assays can be shown for the appropriate quantification of gene copy numbers of hydrogen producing microorganisms in fermentative sludge. The intercept and slope average values was used to calculate the amount of targeted DNA within the samples. The hydrogen producing microbial communities and hydrogenase gene expression was reported as DNA copy numbers per nanogram of genomic DNA from fermentation samples.

Quantitative data shown in Figure 24 and 25 demonstrates the QPCR assay results according to the gene copy number from samples of Raw sludge (S1), iron optimization (S2) experiment and magnetite optimization experiment (S3) using each primer set. *Clostridium* spp. was predominant in all samples demonstrating the highest copy number of 6.23×10^5 in sample 3 containing magnetite nanoparticles whilst the lowest concentration was found in the heat treated raw sludge (1.1×10^5) and the second highest concentration displayed in sample 2 (1.43×10^5) supplemented with iron.

The concentration of Enterobacteriaceae was significantly lower in samples 1 and 2 however showed a substantial increase (1.30×10^5) in sample 3. The progressive increase in the levels of expression of hydrogenase gene (hydA) from the heat pretreated sludge was shown in Figure 24. The number of copies of hydA expressed/ ng DNA was monitored in all three samples to confirm the activity of hydrogen producing bacteria. The highest hydrogenase activity was found in sample 3 displaying a hydA copy number of 8.72×10^2, followed by sample 2 with a copy number of 2.66×10^2 and the least in sample 1 as expected.

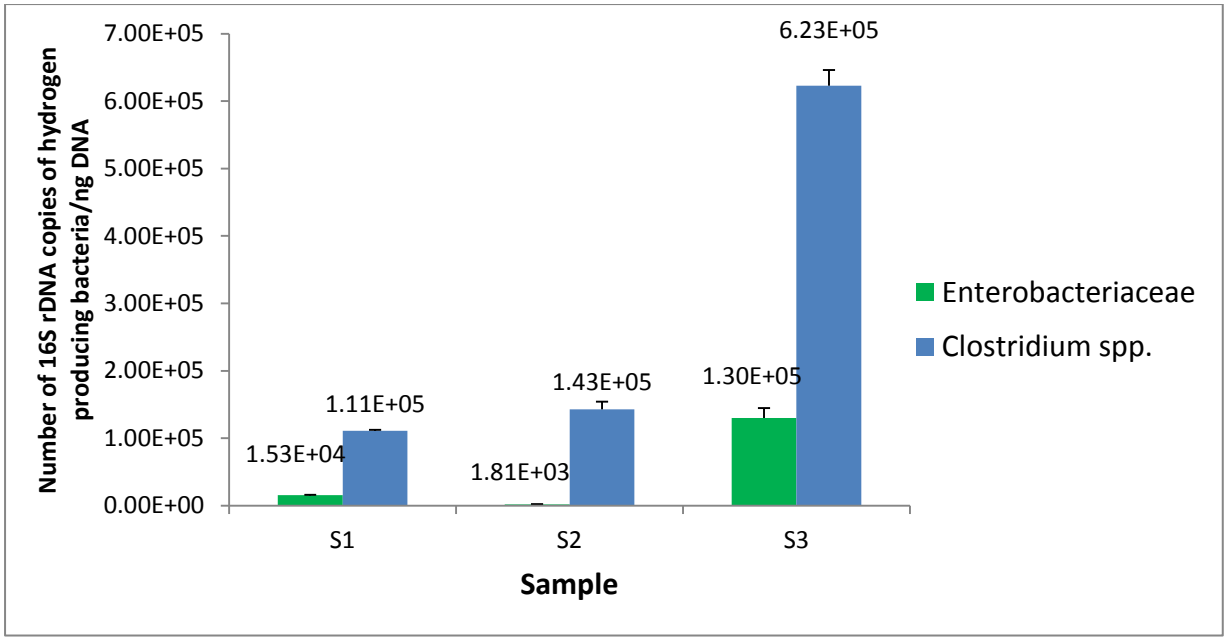


Figure 24. Comparison of hydrogen producing bacterial DNA copy numbers of 16S rDNA per nanogram of genomic DNA from optimization experiments in this study. S1 (Raw sludge); S2 (iron optimization experiment) and S3 (Magnetite optimization experiment)

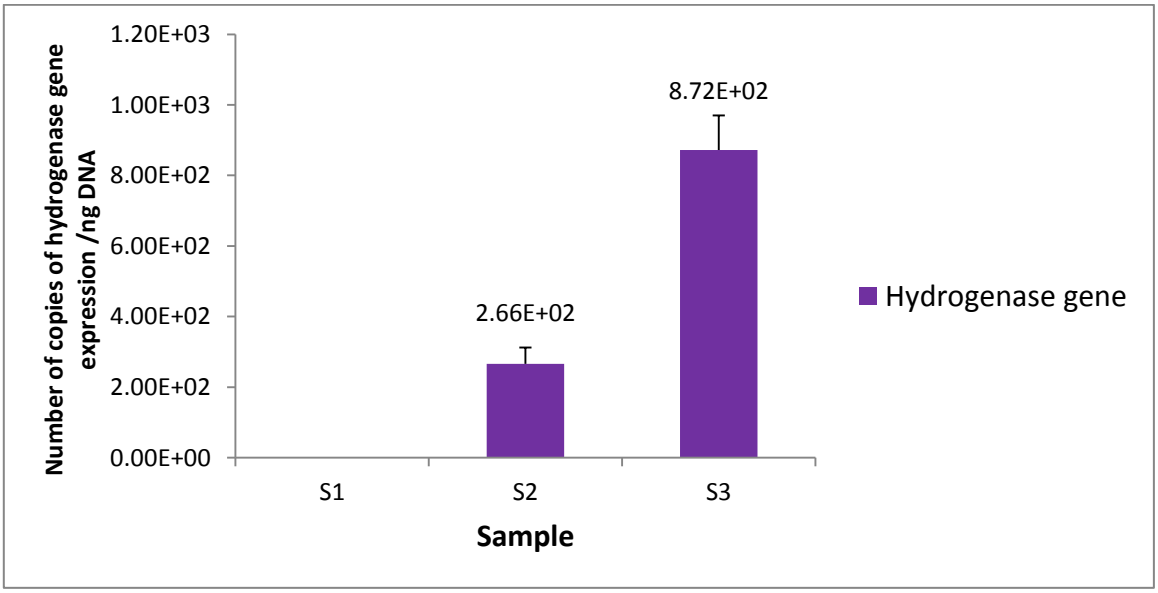


Figure 25. Level of hydrogenase gene S1 (Raw sludge); S2 (Iron optimization experiment) and S3 (Magnetite optimization experiment)

Our finding can be related to previous studies that reported the performance of *Clostridium* spp. is related to the expression of hydA gene. These results can be directly associated with the hydrogen yields produced during the fermentation studies, confirming that higher activity of hydrogen producing bacteria was displayed when magnetite nanoparticles were introduced into the fermentation reaction. Therefore, monitoring the hydrogenase gene expression can be used as a practical index of hydrogen production amongst bacterial communities during dark fermentation (Chang *et al.*, 2006, Wang *et al.*, 2008). Other investigations by Wang have also reported hydrogenase gene amplification by PCR, which have related the hydrogenase gene amplification to *Clostridium* spp. which is common identified in heat treated sludge (Wang *et al.*, 2007a)..

5.4 Conclusions

- The maximum hydrogen production (1.2 mol/mol glucose) was achieved at a pH range of 5-6, a substrate to biomass ratio of 3.5, and iron and magnetite nanoparticle concentration of 200 mg/L.
- Supplement the fermenters with Fe^{2}+ 200 mg L-1 or magnetite nanoparticles concentration 200 mg L-1 improved the HY and SHPR compared to control.
- The generated amounts of biohydrogen at different magnetite nanoparticles concentrations were slightly higher than those at the same Fe^{2}+ concentrations.
- Introduction of nanoparticles into the fermentation reaction could increase the bioactivity of hydrogen-producing microorganisms.
- Clostridium spp., as identified by qPCR, was the most dominant hydrogen producing microorganism during fermentation.
- A positive relationship was confirmed between the known hydrogen producing bacterial consortia and hydrogen yield based on QPCR.
- The highest hydrogenase gene activity was confirmed to be in the culture immobilized on magnetite nanoparticles.

CHAPTER SIX:
GENERAL CONCLUSIONS AND RECOMMENDATIONS

Sugarcane bagasse is an ideal substrate for fermentative biohydrogen production as it is readily available, high in carbohydrates and can be easily degraded. One of the major challenges of using sugarcane bagasse as a substrate for dark fermentation is that the available sugars are locked in the complex cellulose and hemicellulose components within the plant fibre structure. Thus, pre-treatment is needed to disrupt the recalcitrant structure of this lignocellulosic material, by breaking the lignin seal and decreasing crystallinity of cellulose.

The current study focused on the evaluation of different pre-treatment methods for the optimal release sugars from sugarcane bagasse to be used as a fermentation broth for biohydrogen production. The ground sugarcane bagasse samples were subjected to steam (121°C and 1.4 kg/cm$^{3)}$ treatment using different concentrations of acid (0-1%) and alkali (0-3%) solutions. Microwave treatment was also employed using acid (0-1%) and alkali (0-3%) solutions at power 100 W and 180 W, respectively. Statistical method such as design of experiments was selected to determine the individual and interactive effects of time and acid/alkali concentration on sugar yield. The total sugar release was assessed by phenol sulphuric acid method and thereafter the individual sugar composition (High pressure liquid chromatography) in the hydrolysates were analysed to demonstrate the highest carbohydrate content.

Among the different pre-treatment methods analysed in this study, acid treatment (steam) with 0.25% H_2SO_4 for 60 minutes at 121°C yielded the highest quantity (294.4 mg/g) of reducing sugars. SEM analysis of the sample has further shown that during this steam acid treatment, the internal components of the plant structure were completely broken down resulting in optimal sugar release. Additionally, the HPLC analysis confirmed the release of sugars of interest (glucose and xylose) during the steam-acid treatment, while ribose was the primary sugar released during steam-alkali treatment. On the other hand, microwave acid and alkali treatments did not reveal a significant amount of sugar release from the bagasse. Statistical experimental design and surface response methodology were successfully applied to verify the effects of concentration (acid and alkali) and reaction time on sugar yield during different sets of experiments.

Activated (anaerobic) sludge from a domestic and industrial wastewater treatment plant was used as the seed inoculum for the current study. The preliminary microbial characterization of the sludge was carried out using FISH technique. 16S rRNA based species-specific oligonucleotide probes were employed to identify the major hydrogen producing microorganism as well as hydrogen consuming methanogens in raw sludge. Using Fish technique, a higher population of hydrogen producing bacteria was confirmed in domestic sludge compared to the industrial sludge sample. Thermal pre-treatment (heat and sterilization) was then used to selectively eliminate hydrogen consuming methanogenic bacteria from the sludge samples whilst preserving the hydrogen producing bacteria. Both heat and sterilization methods were effective in eliminating hydrogen consuming bacteria. Boiling sludge for 30 minutes was optimal over sterilization treatment due to its negligible destruction of the overall microbial diversity, whilst still conserving the hydrogen producing population.

Based on the sludge pre-treatment results obtained, the study concluded that thermal pre-treatment of sludge is effective for elimination of hydrogen consuming bacteria while assist in selection of hydrogen producing consortium from a mixed sludge consortia. In addition, the FISH techniques were efficient in detection and differentiation microbes from treated and untreated sludge samples. Based on FISH results, the heat treatment at 30 minutes was favoured over sterilization treatment due to its negligible destruction of the overall microbial diversity, whilst still conserving the hydrogen producing population. During the period of experimentation, hydrogen producing *Clostridium* sp. was the most prominent cluster identified. These results aided in selecting the appropriate pre-treatment method for development of seed inoculum for the reactor.

Dark fermentation of the hydrolysate was carried out in batch reactions to determine the effect of pH, substrate to biomass, iron and magnetite nanoparticles on hydrogen production. Modified Gompertz model was used to fit the cumulative hydrogen production in each batch experiment. Hydrogen produced resulted in an optimal yield of 1.2 mol/mol glucose. A pH range of 5-6 and substrate to biomass ratio of 3.5 had displayed the highest production of hydrogen during operational optimization studies. Addition of iron and magnetite nanoparticles to the fermentation broth at a concentration of 200mg/L, enhanced hydrogen production. Scanning electron microscopy was used to confirm the interaction of iron and

magnetite nanoparticles with the microbial consortium within the reactor. Further, QPCR was able to identify the most dominant hydrogen producing microorganism during fermentation. Quantitative data showed the QPCR assay results according to the gene copy number from samples of Raw sludge (S1), iron optimization (S2) experiment and magnetite optimization experiment (S3) using each primer set. *Clostridium* spp. was predominant in all samples demonstrating the highest copy number of 6.23×10^5 in sample 3 containing magnetite nanoparticles whilst the lowest concentration was found in the heat treated raw sludge (1.1×10^5) and the second highest concentration displayed in sample 2 (1.43×10^5) supplemented with iron. The highest hydrogenase gene activity was confirmed to be in the samples supplemented with magnetite nanoparticles (8.72×10^2), followed by sample 2 with a copy number of 2.66×10^2 and the least in sample 1 as expected.

Recommendations

- As observed in this study, low concentrations of reducing sugars were obtained from sugarcane bagasse pre-treatment. Using a combination of other waste materials such as sewage sludge along with sugarcane bagasse may potentially improve the substrate limitation and will contribute to the sustainable waste sludge management in South Africa.
- Identification of waste material that requires minimal pre-treatment would make the dark fermentation process cost effective.
- Sequential acid and alkaline process to release higher amounts of sugar from sugarcane bagasse wasn't studied; and that will be the focus of our future work.
- Using a combination of continuous dark and photo fermentation would improve the overall hydrogen yield

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APPENDICES

APPENDIX ONE (Chapter three)

Table 10. Measured and simulated (response surface) sugar yield of steam-acid hydrolysis

Condition		Steam H_2SO_4		Steam HCl		Response surface plot*		
Conc. (%)	Time (min)	Measured sugar yield (mg/g)	Predicted sugar yield (m/g)	Measured sugar yield (mg/g)	Predicted sugar yield (m/g)		Steam H_2SO_4	Steam HCl
0	30	128.3	116.8	128.301	134.4	A	-101.93	-47.67
0.25	30	143.9	178.1	199.2	167.1	B	9.80	8.17
0.5	30	211.2	212.2	130.899	174.2	C	321.70	173.61
0.75	30	232.0	219.0	160.8	155.6	D	-0.73	0.30
1	30	209.2	198.6	123.3	111.2	E	-0.08	-0.07
0	60	170.4	184.7	170.4	190.6	F	-218.02	-205.67
0.25	60	294.4	240.6	275.199	225.6	R^2	0.8429	0.6826
0.5	60	275.5	269.2	223.299	234.9			
0.75	60	244.2	270.5	192.3	218.5			
1	60	225.1	244.6	184.599	176.3			
0	90	96.4	101.9	96.399	120.9			
0.25	90	143.3	152.3	199.5	158.2			
0.5	90	187.2	175.4	159.999	169.7			
0.75	90	151.1	171.3	139.5	155.5			
1	90	162.8	140.0	124.5	115.6			

* $Y = A + B \times X_1 + C \times X_2 + D \times X_1 X_2 + E \times (X_1)^2 + F \times (X_2)^2$

Table 11. Measured and simulated (response surface) sugar yield of steam-alkali hydrolysis

Condition		Steam NaOH		Steam $Ca(OH)_2$		Response surface plot*		
Conc. (%)	Time (min)	Measured sugar yield (mg/g)	Predicted sugar yield (m/g)	Measured sugar yield (mg/g)	Predicted sugar yield (m/g)		Steam NaOH	Steam $Ca(OH)_2$
0	60	122.8	190.0	122.8	157.6	A	679.87	254.75
1	60	304.0	343.6	188.6	160.7	B	-10.90	-2.11
2	60	578.7	506.3	182.8	176.1	C	252.92	14.03
3	60	712.4	677.9	204.2	204.0	D	-1.73	-0.29
0	120	160.7	28.2	160.7	119.0	E	0.05	0.01
1	120	119.3	77.9	84.1	104.9	F	4.51	6.20
2	120	73.6	136.7	103.4	103.1	R^2	0.8806	0.7227
3	120	93.7	204.5	92.6	113.8			
0	180	139.6	194.2	139.6	138.9			
1	180	106.4	140.1	77.5	107.6			
2	180	117.8	95.1	104.6	88.6			
3	180	124.7	59.1	95.5	82.1			

* $Y = A + B \times X_1 + C \times X_2 + D \times X_1 X_2 + E \times (X_1)^2 + F \times (X_2)^2$

Table 12. Measured and simulated (response surface) sugar yield of microwave-acid hydrolysis

Condition		**Microwave H_2SO_4**		**Microwave HCl**		**Response surface plot***		
Conc. (%)	**Time (min)**	**Measured sugar yield (mg/g)**	**Predicted sugar yield (m/g)**	**Measured sugar yield (mg/g)**	**Predicted sugar yield (m/g)**		**Micro wave H_2SO_4**	**Micro wave HCl**
0	15	67.0	75.3	67.0	69.3	A	-36.40	169.09
0.25	15	103.4	85.4	106.6	99.0	B	9.03	-10.10
0.5	15	118.6	104.0	137.1	116.0	C	73.55	174.64
0.75	15	138.5	131.0	99.5	120.4	D	-3.34	-2.04
1	15	134.7	166.5	106.6	112.1	E	-0.11	0.23
0	30	117.1	139.4	57.1	73.1	F	67.69	-101.24
0.25	30	119.4	137.0	88.6	95.1	R^2	0.5916	0.9237
0.5	30	160.4	143.1	115.4	104.5			
0.75	30	150.1	157.6	92.9	101.2			
1	30	210.7	180.6	105.1	85.3			
0	45	198.6	156.0	198.6	180.4			
0.25	45	101.5	141.0	184.3	194.7			
0.5	45	148.1	134.6	192.7	196.5			
0.75	45	115.2	136.6	186.4	185.5			
1	45	151.9	147.1	157.1	162.0			

* $Y = A + B \times X_1 + C \times X_2 + D \times X_1 X_2 + E \times (X_1)^2 + F \times (X_2)^2$

Table 13. Measured and simulated (response surface) sugar yield of microwave-alkali hydrolysis

Condition		**Microwave NaOH**		**Microwave $Ca(OH)_2$**		**Response surface plot***		
Conc. (%)	**Time (min)**	**Measured sugar yield (mg/g)**	**Predicted sugar yield (m/g)**	**Measured sugar yield (mg/g)**	**Predicted sugar yield (m/g)**		**Microwave NaOH**	**Microwave $Ca(OH)_2$**
0	60	90.1	115.4	90.1	90.9	A	102.63	-113.42
1	60	97.6	76.1	104.3	108.0	B	0.26	4.45
2	60	70.4	71.9	85.4	106.8	C	-51.76	35.33
3	60	107.9	102.6	113.2	87.2	D	-0.08	-0.15
0	120	165.4	122.7	165.4	169.7	E	0.00	-0.02
1	120	48.0	78.5	221.2	177.7	F	17.50	-9.16
2	120	70.9	69.2	156.0	167.4	R^2	0.5465	0.7361
3	120	81.0	94.9	110.9	138.7			
0	180	110.6	124.6	110.6	122.9			
1	180	74.3	75.4	134.2	121.8			
2	180	70.9	61.1	82.9	102.4			
3	180	87.1	81.8	83.9	64.6			

* $Y = A + B \times X_1 + C \times X_2 + D \times X_1 X_2 + E \times (X_1)^2 + F \times (X_2)^2$

Table 14. Soluble fraction of sugarcane bagasse at different conditions of steam-acid, steam-alkali, microwave-acid and microwave-alkali

Hydrolysis method	**Condition**	**Xylose (g/L)**	**Glucose (g/L)**	**Mannose (g/L)**	**Ribose (g/L)**
Steam-acid	60 min 0.25% H_2SO_4	1.851	0.701	0	0
	60 min 0.5% H_2SO_4	2.329	1.049	0	0
	60 min 0.75% H_2SO_4	2.14	1.051	0	0
	60 min 1% H_2SO_4	1.595	0.866	0	0
	30 min 0.25% HCL	1.88	0.752	0	0
	90 min 0.25% HCL	1.979	0.944	0	0
Steam-alkali	60 min 2% NaOH	1.552	0.631	0	0
	60 min 3% NaOH	0	0	0	0.140
	120 min 1% NaOH	0	0	0	0.181
	120 min 2% $Ca(OH)_2$	0	0	0	0.135
	180 min 2% $Ca(OH)_2$	0.528	0.360	0	0
Microwave-acid	15 min 0.75% H_2SO_4	0.563	0.360	0	0
	30 min 0.5% H_2SO_4	0.426	0.283	0	0
	30 min 1% H_2SO_4	0.644	0.415	0	0
	15 min 0.5% HCL	0.352	0.233	0	0
	30 min 0.5% HCL	0.403	0.417	0	0.000394
	45 min 0.5% HCL	0.625	0.460	0	0.00883
Microwave-alkali	60 min 3% NaOH	0.017	0.110	0	0
	60 min 3% $Ca(OH)_2$	0.345	0.38	0	0.046
	120 min 1% $Ca(OH)_2$	0	0.148	0.076	0.042
	180 min 1% $Ca(OH)_2$	0	0.161	0.135	0.012

APPENDIX TWO (Chapter Five)

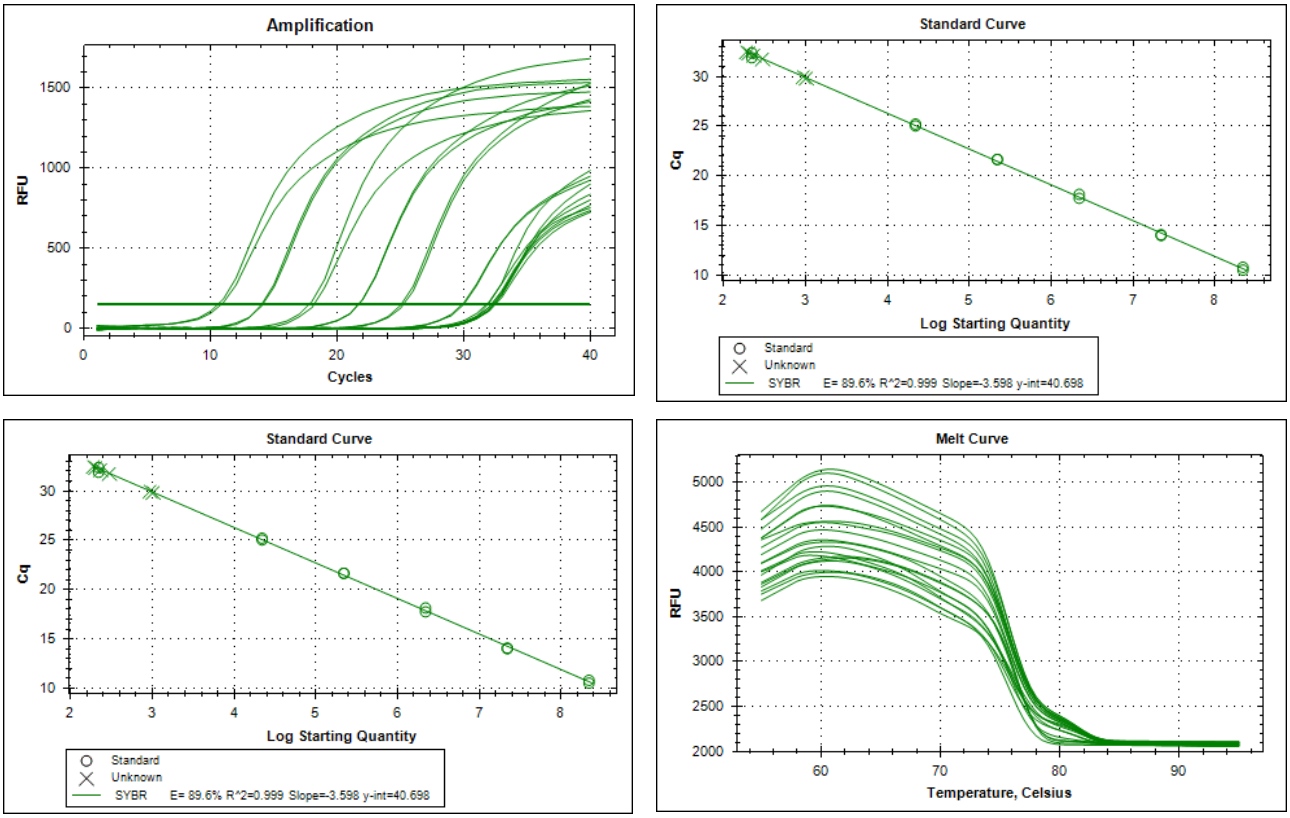

Figure 26. Quantitative polymerase chain reaction standard curve results for hydrogenase gene amplification.

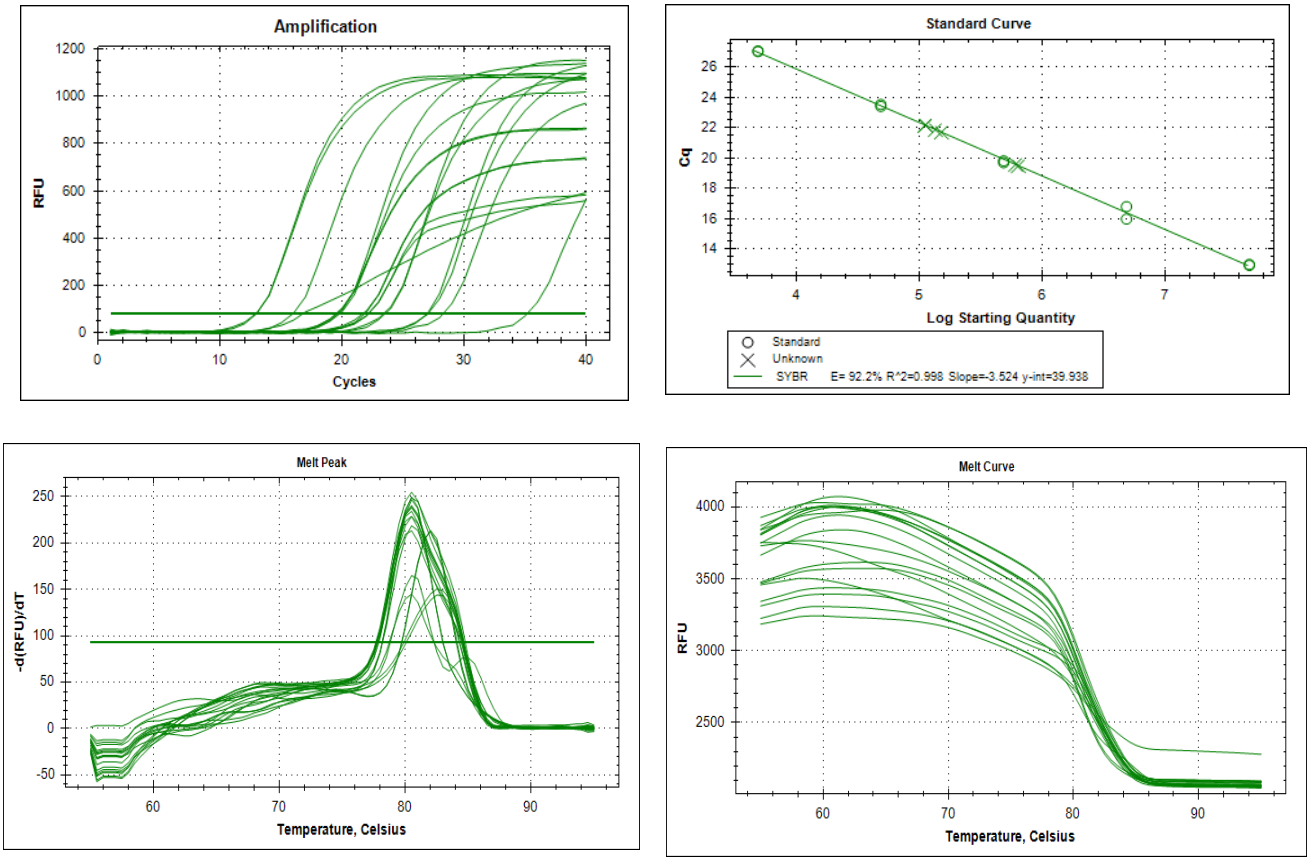

Figure 27. Quantitative polymerase chain reaction standard curve results for *Clostridium* spp. amplification.

Figure 28. Fermentative biohydrogen production experiments at laboratory scale.

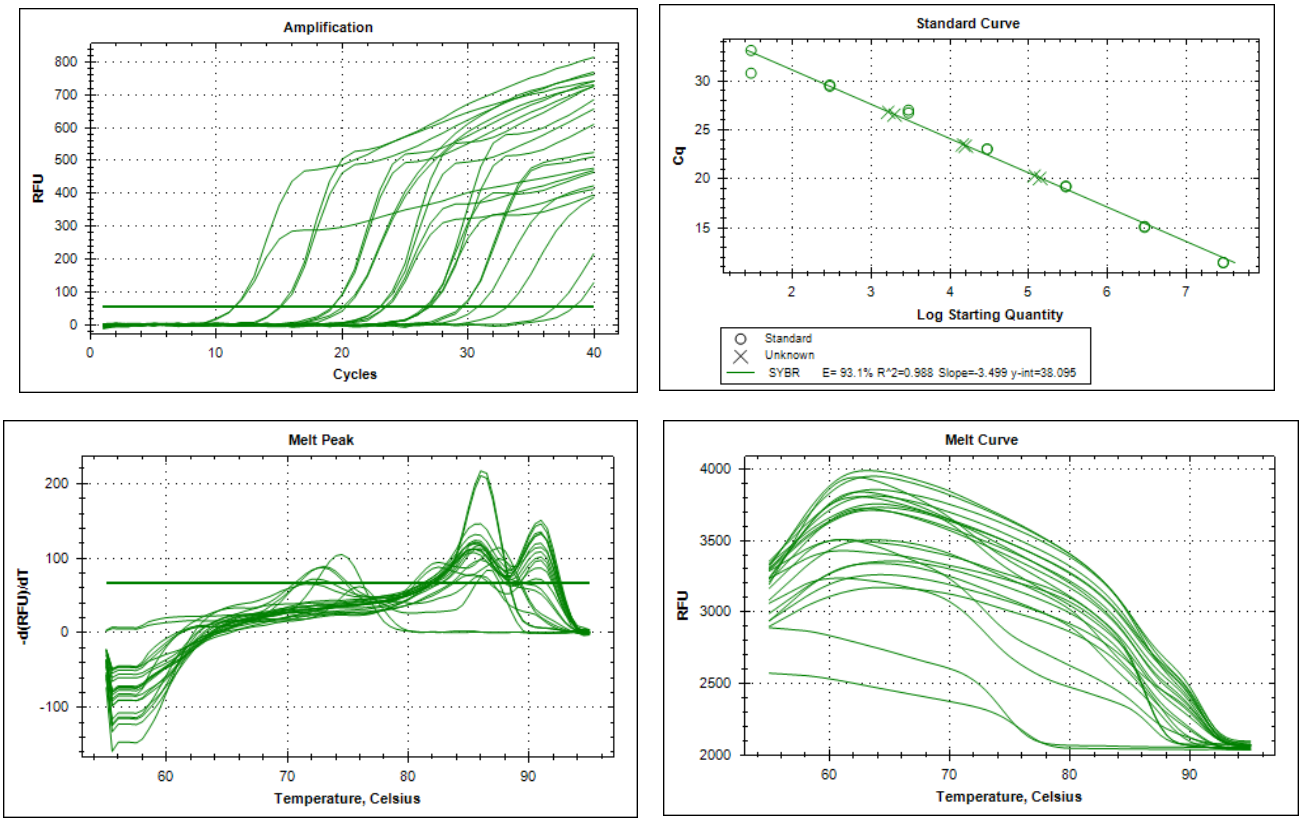

Figure 29. Quantitative polymerase chain reaction standard curve results for Enterobacteriaceae amplification.