Enhancement of biohydrogen production from the aquatic weed *Pistia stratiotes* through a dark fermentation process

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APPROVAL

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DECLARATION

I hereby declare that this thesis entitled "**Enhancement of biohydrogen production from the aquatic weed *Pistia stratiotes* through dark fermentation process**"

1. Is my original work and has not been submitted for a degree at any other university, its only prior publication was in the form of a journal article.

2. I further declare that a detailed reference list has been provided on all cited literatures and resources.

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ABSTRACT

Aquatic weeds are well known for their fast growth rate and high carbohydrate content that can be easily hydrolysed into fermentable sugars. This study was aimed at the utilization of an indigenous aquatic weed, *Pistia stratiotes* for biohydrogen production through the dark fermentation process. Characterization of the biomass, effect of pre–treatment methods on biomass hydrolysis, effect of reactor operational conditions and type of inoculum on enhancing hydrogen production potential of *P. stratiotes* was assessed. Physical and chemical pre–treatments were employed on *P. stratiotes* biomass to increase digestibility and to achieve high conversion rates of fermentable sugars. The highest sugar yield of 139± 0.8 mg/g was obtained when the oven dried biomass was subjected to H_2SO_4 (2.5%) pre–treatment followed by autoclaving at 121°C for 30 min. Biohydrogen production under different operational conditions was thereafter optimized using One–factor–at–a–time (OFAT) batch experiments in 120 mL serum bottles. A maximum hydrogen yield (HY) of 2.46 ± 0.14 mol-H_2/mol-glucose (3.51 ± 0.20 mg-H_2/g-dry weight) and 2.75 ± 0.07 mL h^{-1} hydrogen production rate was observed under optimized conditions (pH 5.5, Temp 35°C, S/X: 1.0 g-COD/g-VSS and HRT 8 h). The organic mass balance (92 – 96%) and electron–equivalent balance (92 – 98%) further indicated the reliability of the obtained fermentation data. Assessment of microbial activity was achieved using molecular techniques such as quantitative polymerase chain reaction (qPCR) targeting both 16s rRNA (of *Clostridium* spp., *Bacillus* spp., and *Enterobacter* spp.) and the functional hydrogenase gene (hydA). The highest gene activity of hydrogenase was noted at pH of 5.5 with 2.53×10^4 copies/ng-DNA compared to low pH: 4.5 (6.95×10^3 copies/ng-DNA) and high pH: 8.5 (7.77×10^3 copies/ng-DNA). A similar trend was also observed for the species containing a highly active hydrogenase (i.e. *Clostridium* spp., *Bacillus* spp., and *Enterobacter* spp.). During the optimum reactor conditions, three hydrogen producing bacterial strains *Bacillus cereus* and

Enterobacter cloacae were successfully isolated. These isolates were used as inoculums for the pure culture studies and achieved HYs of 2.2, 1.10 and 1.97 mol-H_2/mol-glucose respectively under optimized fermentation conditions. However, the thermally treated mixed culture displayed a marginally higher HY (2.46 mol-H_2/mol-glucose) compared to the pure culture used alone. Furthermore, the cost estimation indicated a potential and economically feasible for biotransformation of *P. stratiotes* to hydrogen energy. In conclusion, the results from this study has revealed the potential of employing *P. stratiotes* biomass for biohydrogen production. The results also indicated the importance of employing suitable pre–treatment methods, operating conditions as well as inoculum types for enhanced hydrogen production using *P. stratiotes.*

PREFACE

The outputs of the Masters qualification include:

Publications

- MTHETHWA, N. P., NASR, M., BUX, F. & KUMARI, S. 2018. Utilization of *Pistia stratiotes* (aquatic weed) for fermentative biohydrogen: Electron-equivalent balance, stoichiometry, and cost estimation. *International Journal of Hydrogen Energy,* 43**,** 8243-8255

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HPLC:	High pressure liquid chromatography
SEM:	Scanning electron microscopy
TS:	Total solids
COD:	Chemical oxygen demand
FISH:	Fluorescent In–Situ Hybridization
FAM:	Carboxy-fluorescein
DAPI:	4’,6-diamidino-2-phenylindole
HRT:	Hydraulic retention time
S/X:	Substrate-to-biomass ratio
PCR:	Polymerase Chain Reaction
qPCR:	Quantitative Polymerase Chain Reaction
HY:	Hydrogen yield
HPR:	Hydrogen production rate
H_2:	Hydrogen

CHAPTER I

INTRODUCTION

Environmental pollution has urged the scientific community to look for alternate and sustainable sources for energy production. Hydrogen gas (H_2) is considered as a potential green energy carrier that can meet the increasing global energy demand because it is renewable and produces only water during combustion (Møller et al., 2017, Mitsushima and Hacker, 2018). Hydrogen has a higher energy content per unit weight (122 – 142 kJ/g) compared to gasoline (47.4 kJ/g), methane (56 kJ/g) and biodiesel (37.0 kJ/g) (Vi et al., 2017). Hydrogen produces only H_2O during combustion and can be utilized in fuel cells and vehicle engines (Bharathiraja et al., 2016, Dimitriou et al., 2018). In addition, H_2 has been widely involved in several processes such as petroleum refining, fertilizer production, and ammonia manufacturing (Łukajtis et al., 2018).

Hydrogen can be generated using multiple methods, viz., physico–chemical (steam reforming, electrolysis, thermolysis) and biological (Nikolaidis and Poullikkas, 2017, Ghasemzadeh et al., 2018). The physico–chemical methods are efficient and fast, however, they employ large quantities of heavy oil, fossil materials, electricity, wind, hydro, and geothermal energy, leading to increasing the energy input and environmental pollution (Argun et al., 2017). Biological methods for H_2 production are generally considered as environmentally friendly and can be conducted under ambient operating temperatures and atmospheric pressures with low energy input (Qiu et al., 2016, Mishra et al., 2017b). Hydrogen production through biological route is currently achieved through different processes. This includes 1) direct bio–photolysis using green algae, 2) indirect bio–photolysis using cyanobacteria, 3) photo fermentation using photosynthetic bacteria and 4)

dark fermentation using facultative/anaerobic bacteria (Nikolaidis and Poullikkas, 2017, Aslam et al., 2018, Haron et al., 2018). Photo fermentative H_2 production is carried out by photoheterotrophic bacteria feeding on carbon rich substrates and volatile fatty acids in an environment deprived of nitrogen and oxygen (Ghosh et al., 2017). This process depends directly on sunlight energy, water, and acetic acid or butyric acid (Rai, 2016).

Dark fermentation is considered as an ideal and efficient method for treating large quantities of organic wastes (Łukajtis et al., 2018, Rafieenia et al., 2018b, Silva et al., 2018b). In this process, microorganisms ferment substrates that are rich in carbohydrates to hydrogen (Singh and Wahid, 2015). This trend results in achieving a net positive energy balance (i.e. producing hydrogen) and in parallel, solves environmental pollution problems (i.e. wastes removal) (Kumar et al., 2014). The major advantage of dark fermentation is that it is independent of light and has an adequate conversion efficiency (Shaterzadeh and Ataei, 2017). The by–products from dark fermentation can also be utilized for photo fermentation as a second step to improve the hydrogen yield (HY) (Chandrasekhar et al., 2015, Zagrodnik and Łaniecki, 2017). The sequential dark–photo fermentation also allows the confines of the processes when individually operated to be overcome (Ghosh et al., 2018, Zhang et al., 2018). Hence, from the perspectives of both energy and environmental concerns, anaerobic fermentation is a feasible and preferable approach over other biological methods.

Dark fermentation is carried out by a group of hydrolytic and acid forming bacteria involving three of four anaerobic digestion steps viz., hydrolysis, acidogenesis, and acetogenesis (Cardoso et al., 2014, Adekunle and Okolie, 2015, Jaseena and Sosamony, 2016). In a light deprived environment, the activity of hydrogenase enzymes results in the oxidation of organic substrates and the release of excess electrons (Ghimire et al., 2015a). The disposed

electrons are neutralized by protons that act as electron acceptors, consequently generating H_2 (Rezania et al., 2017). Each mol of glucose is accompanied by 4 mol of H_2 when acetic acid is the main fraction in the metabolic pathway, whereas 2 mol of H_2 per mol-glucose can be obtained for a final product of butyric acid (Ginkel et al., 2001, Nikolaidis and Poullikkas, 2017).

The dark fermentation process is highly sensitive to the variations in operational conditions such as pH, temperature, hydraulic retention time (HRT), substrate to biomass (S/X) ratio, inoculum type etc. (Jaseena and Sosamony, 2016, Reddy et al., 2017, Zagrodnik and Łaniecki, 2017). The control of pH in the reactor is necessary for the prevention of the growth of methanogens and other unwanted microbes (Wang and Yin, 2017). Similarly, the control of S/X and HRT plays a very significant role in respect to obtaining optimum microbial growth and hydrogen production efficiency (Mohammadi et al., 2012). Likewise, variation in temperature can directly influence hydrogen production efficiency by affecting the growth of microbial species, their activity as well as the metabolic products (Wongthanate and Chinnacotpong, 2015, Argun et al., 2017). It has been reported that thermophilic (45°C – 55°C) conditions improve the hydrogen production rate compared to mesophilic (30°C – 40°C) (Mohammadi et al., 2012). However, a sudden elevation to extremely high or low temperature can hinder the rate of biohydrogen production (Mohammadi et al., 2012).

Pure cultures of *Bacillus* spp., *Enterobacter* spp., and *Clostridium* spp. have shown their H_2 production potential from various feedstock in dark fermentation studies (Palomo-Briones et al., 2017, Rafieenia et al., 2018a). However, pure cultures are limited by the requirement of stringent and sterile growth conditions leading to increased operational costs (Pachapur

et al., 2015, Fatehizadeh et al., 2018, Łukajtis et al., 2018). Mixed cultures, on the other hand, carry a wide range of microbial consortia with varying H_2 production potential under different environmental conditions (Fatehizadeh et al., 2018). However, mixed cultures such as anaerobic sludge should be subjected to pre–treatment process before inoculation in fermenters, to enhance the H_2 producing communities and eliminate the hydrogen consumers and H_2 competing microorganisms such as homoacetogens, methanogens, and lactic acid bacteria (Nasr et al., 2013, Singh et al., 2015, Zheng et al., 2016, Kumari and Das, 2017). Various pre–treatment methods such as pH control, heat shock, and freezing–and–thawing have been tried for removing H_2 consuming bacteria from the seed inoculum (Dessì et al., 2018). The major advantage of H_2 producers are their ability to form spores which can withstand the harsh pre–treatment methods (Wang and Yin, 2017, Rafieenia et al., 2018a). The use of extremely high and low pH ranges is considered to affect the enzyme activity and nutrient adsorption of the methanogens (Bundhoo et al., 2015, Rafieenia et al., 2018a). These methanogens do not have the ability to form the protective spores during harsh conditions as compared to H_2 producers (Bundhoo et al., 2015, Rafieenia et al., 2018a).

Different substrates, rich in carbohydrates can be used for biohydrogen production such as pure glucose/starch mixtures, first generation fuel crops (wheat, sugar beets, and sugarcane) as well as second generation biomass (Sutthipattanasomboon and Wongthanate, 2017, Kumar et al., 2018b). The second generation biomass includes industrial waste (e.g. cheese whey and brewery waste sludge), agricultural residues (e.g. corn stalk, bagasse and wheat straw), and aquatic weeds (Wong et al., 2018). Lignocellulosic materials are a renewable source of second generation biomass, they are cost effective, and abundant (Kumar et al., 2017). These are however composed of a complex structure with cellulose, hemicellulose and lignin (Nissilä et al., 2014). Therefore prior to fermentation the substrate is hydrolysed

by physical, chemical and biological pre–treatment processes for ease in digestibility (Rafieenia et al., 2018a, Wang and Yin, 2018).

This study focused on assessing the potential of using the hydrolysates of aquatic weed, *Pistia stratiotes* for biohydrogen production. Invasive aquatic plants, to South Africa, such as water hyacinth (*Eichhornia crassipes*), mosquito fern (*Azolla filiculoides*), cabomba (*Cabomba caroliniana*) and water lettuce (*Pistia stratiotes*) are categorized by their high moisture contents and reproductive capabilities (Chuang et al., 2011). The increased invasive rates of these plants negatively affect water quality, natural resource cycling, ecological functioning, and biodiversity (O'Sullivan et al., 2010, Chamier et al., 2012, van Wilgen et al., 2017). Among these, *P. stratiotes* is a free-floating aquatic plant that can completely shield waterways and dams by forming dense mats in a shorter period. This plant is therefore considered as one of the 'Big Bad Five' invasive free-floating aquatic plants with an increasing population from 19% in 2008 to 26% in 2015 (Hill and Coetzee, 2017). In recent years, Diep River in Cape Town and Sunset Dam in Kruger National Park became heavily infested with *P. stratiotes*, which adversely affects the economic and environmental development of South Africa (van Wilgen et al., 2017). Several studies have reported the use of *P. stratiotes* for biofuel production mainly following CH_4, ethanol (C_2H_5OH), and CO_2 production (Namadi, 2013, Rezania et al., 2015, Gusain and Suthar, 2017).

To date, only one study has investigated the bioconversion of water lettuce (*Pistia stratiotes*) into H_2 via a photo fermentation process (Corneli et al., 2017). In their study the biomass was subjected to hydrolysis, and acidogenesis phases and the obtained organic acids were utilized as a substrate by Purple Non Sulphur Bacteria (PNB), namely *Rhodopseudomonas palustris* 42OL and *Rhodopseudomonas palustris* CGA676 as the inoculum. However, the

energy consumption (150 μmol/photons/m295 /s) during photo fermentation could increase the operating cost of the system and result in an expensive H_2 production process. This study therefore focused on the evaluation of the hydrolysates of *P. stratiotes* fermentative bio hydrogen production through dark fermentation. The effect of pre–treatment methods, operational conditions as well as type of inoculum on enhancement of hydrogen production potential of *P. stratiotes* was also assessed. The total cost of the fermentation system including the amortization cost of investment and operating cost was assessed in order to assess the feasibility of hydrogen production from *P. stratiotes* through dark fermentation.

AIM AND OBJECTIVES

Aim

To assess the feasibility of using the biomass of the indigenous aquatic weed, *Pistia stratiotes* for enhanced biohydrogen production through the dark fermentation process.

Objectives

- To evaluate pre–treatment methods to hydrolyse the biomass of *P. stratiotes* via characterization of the hydrolysate.
- To determine the feasibility of anaerobic digestion of the hydrolysates of *P. stratiotes* for biohydrogen production using thermally treated activated sludge as the inoculum.
- To optimise the operational conditions (S/X ratio, HRT, temperature and pH) for increased hydrogen production rate on the selected hydrolysates based on chemical and microbiological analysis.
- To identify the dominant microbial consortia during optimum hydrogen production for the development of a potential hydrogen producing microbial cocktail.
- To estimate the costs for the production of hydrogen from *Pistia stratiotes* through the dark fermentation process using thermally treated sludge as an inoculum.

CHAPTER: I I

LITERATURE REVIEW

2.1 Advantages of hydrogen gas as a renewable energy

The world's reliance on coal, natural gas and oil for energy supply leads to ongoing climate change challenges (Sterk et al., 2016). These energy sources contribute to an increase in emissions of greenhouse gases such as carbon monoxide (CO), carbon dioxide (CO_2) and sulfur dioxide (SO_2) (Dincer and Acar, 2015, Yang and Wang, 2018). Hydrogen (H_2) has been considered as a tremendous alternative source of energy that produces up to 122 kJ more energy compared to conventional energy sources (Vi et al., 2017). Hydrogen is one of the most abundant and common element in the universe (Blaszczak and Boxe, 2015). It is renewable, nontoxic and can also be found in combination with other elements (Kalamaras and Efstathiou, 2013a), additionally when successfully separated, it can be used in motor vehicle industries, chemical industries and for electricity generation (Dutta, 2014).

2.2 Methods for hydrogen production

Pure H_2 can be recovered by thermochemical, electrochemical and biological processes (Abdalla et al., 2018, Wang and Yin, 2018). Thermochemical processes, such as steam reforming, pyrolysis, steam gasification and gasification, requires high temperatures to produce H_2 from water or hydrocarbons (Demirbas, 2016). Thermochemical methods are productive but the efficiency is limited by high operational cost (Kalamaras and Efstathiou, 2013b). Steam reforming is a frequently used method due to its high H_2 productivity and efficiency (Balat, 2008, Kaiwen et al., 2018). Electrochemical process on the other hand is one of the simplest processes, yet not economically feasible, as it generates H_2 gas from chemical reactions with 50 – 60% energy consumption (Dincer and Joshi, 2013, Santos et

al., 2013). Although these processes are highly efficient, their sustainability for energy generation depends on the type of feedstock used (Pandu and Joseph, 2012, Dutta, 2014). Currently, approximately more than 84% H_2 is still produced from fossil fuels (Balat, 2008, Pandu and Joseph, 2012, Kalamaras and Efstathiou, 2013b, Jaseena and Sosamony, 2016). About 50% of this is from steam reforming (Kalamaras and Efstathiou, 2013b), which calls for more focus on clean, renewable and sustainable methods such as biological processes.

Hydrogen production through biological processes is carried out by different group of microorganisms using biomass resources such as plant materials, agroindustry wastes, as well as water through different processes (Gürtekin, 2014). This include direct and indirect photolysis (Sen et al., 2008), photo fermentation (Ghosh et al., 2018) and dark fermentation (Chen et al., 2008, Akroum-Amrouche et al., 2013, Bundhoo et al., 2015). The major advantages of biological processes include: 1) the ability to operate under mild temperatures and pressure, 2) the ability to utilize a broad range of cheap feedstock and 3) the processes do not produce harmful byproducts that can contribute to the increase in greenhouse gases (Varrone et al., 2015, Wang and Yin, 2018).

Direct photolysis uses algae (such as *cyanobacteria and algae*) to split water molecules to H_2 in the presence of sunlight, and indirect photolysis uses the nitrogenase and hydrogenase enzymes to carry out the process (Gürtekin, 2014). Bio photolysis methods are reported to have a fast production rate however, they are limited by: 1) their dependence on sunlight, 2) inhibition of hydrogenase enzymes by oxygen, and 3) the requirement for specific apparatus for photosynthesis, with efficiency in capturing solar energy (Hallenbeck and Benemann, 2002).

Dark and photo fermentation are the promising alternatives for biological H_2 production (Singh and Wahid, 2015). During photo fermentation, purple non–sulfur bacteria (PNB) utilize light as the energy source and convert the organic acids into H_2 (Wang and Wan, 2009). PNB can produce adenosine triphosphate (ATP) molecules under anoxic conditions and permit the flow of energy electrons, causing proton reduction to H_2 by the action of nitrogenase enzyme (Ghosh et al., 2017). Photo fermentation bacteria can further utilise organic acids produced by dark fermentation to maximise H_2 yield (Rai, 2016). Dark fermentative H_2 production process on the other hand has added advantages over photo fermentation process as it is independent of light and provides faster hydrogen production rate (Lalman et al., 2013). Additionally, integration of the dark and photo fermentation bacteria co–exist in one system leading to reduced production cost (Ghosh et al., 2018). The process is however limited by the requirement of continuous light and the requirement for high amount of conversion energy (Lalman et al., 2013, Rai, 2016).

2.3 Dark fermentation process

Dark fermentation is the production of H_2 from organic compounds (high in sugar) through an anaerobic process (Gürtekin, 2014). Dark fermentation can utilize a wide variety of renewable carbon sources such as plant materials, crop residues, or wastewater effluent (Lalman et al., 2013, Ghosh et al., 2018). The process is formed by diverse group of bacteria involving series of biochemical reactions comparable to anaerobic digestion (Łukajtis et al., 2018). Different group of bacteria (hydrolytic, acidogenic, acetogenic and homoacetogenic) work together to obtain a stable fermentation through acclimatization, conversion and disintegration of the carbon–based matter into H_2 and CH_4 (Jaseena and Sosamony, 2016, Bajpai, 2017). The fermentative hydrolytic microorganisms hydrolyze complex organic polymers to monomers, which are further converted to a mixture of lower molecular organic

acids and alcohol by acidogenic microorganisms (Silva et al., 2018a). Molecular H_2 production occurs via a reversible reduction reaction of protons to H_2, catalysed by 3 possible enzymes namely nitrogenase, Fe-hydrogenase and NiFe-hydrogenase depending on the microorganisms available (de Sá et al., 2011, Hansen and Perner, 2016).

There are two primary biochemical pathways Eq. (1–2). (pyruvate–ferredoxin oxidoreductase and pyruvate formate lyase) for H_2 production under anaerobic fermentation. When glucose is used as a substrate, hydrogen producing bacteria convert glucose to pyruvate via the glycolytic pathway producing adenosine triphosphate from adenosine diphosphate and the reduced form of nicotamide adenine dinucleotide (Ghimire et al., 2015). Pyruvate is then converted (oxidation) to acetyl coenzyme A (Acetyl-CoA), CO_2 and H_2 (Eroglu and Melis, 2016, Kumar et al., 2018b). This pathway is catalysed by pyruvate–ferredoxin oxidoreductase (PFOR) and hydrogenase (Eroglu and Melis, 2016). Theoretically at the end of a complete oxidation 12 moles of H_2 should be produced from 1 mole of hexose (Bajpai, 2017). Facultative anaerobic microorganisms such as *Klebsiella* follow the pathway of pyruvate formate lyase (PHFL) whereby it catalyses the conversion of pyruvate to acetyl coA and formate, which is further converted to H_2 and CO_2 (Khanna and Das, 2013). Acetyl can be converted to acetate, butyrate and ethanol depending on the microorganism present and the substrate (Rafieenia et al., 2018b). Theoretically glucose can be degraded by facultative/obligate anaerobes such as *Clostridium* spp. following the PFOR pathway and can produce 4 moles of H_2 per mole of glucose (Clion et al., 2015). Strict anaerobes can theoretically produce 2 moles of H_2 from glucose when following the PFL pathway (Khanna and Das, 2013). When the microorganisms follow the acetate producing pathway, 4 moles of H_2 are produced from 1 mole of hexose and for butyrate producing pathway only 2 moles from 1 mole of glucose (Rafieenia et al., 2018b) (Table 1).

Main biochemical reaction for dark fermentation:

$$\text{Pyruvate} + \text{CoA} \rightarrow \text{Acetyl} - \text{CoA} + \text{Formate}\ \text{(Pyruvate formate lyase)} \quad (1)$$

OR

$$\text{Pyruvate} + \text{CoA} + 2Fd_{(\text{OX})} \rightarrow \text{Acetyl} - \text{CoA} + CO_2 + 2Fd\ (\text{red})\ \text{(pyruvate–ferredoxin oxidoreductase)} \quad (2)$$

Table 1. Metabolic products of dark fermentation

Pathway	Chemical reaction	References
Acetic acid	$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$	(Clion et al., 2015)
Propionic acid	$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$	(Chandrasekhar et al., 2015)
Butyric acid	$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$	(Florio et al., 2017)
Malic acid	$C_6H_{12}O_6 + 2H_2 \rightarrow COOHCH_2CH_2\,OCOOH + CO_2$	(Chandrasekhar et al., 2015)
Ethanol	$C_6H_{12}O_6 \rightarrow CH_3CH_2OH + CO_2$	(Cappai et al., 2015)

2.3.1 Microbiology of dark fermentation

Dark fermentation is a complex fermentation process that involves symbiotic association of different types of bacteria. The major reaction sequences can be divided into four groups which are similar to anaerobic digestion involving 1) Hydrolysis 2) Fermentation 3) Acetogenesis and 4) Methanogenesis (Jaseena and Sosamony, 2016) (Figure 1).

During hydrolysis, hydrolytic enzymes (amylase, lipase cellulase and protease) excreted by fermentative microorganisms (acidogenic bacteria) breaks down the large organic polymers such as starches, cellulose, proteins and fats into soluble organic molecules such as sugars, amino acids, glycerol and long chain fatty acids (Azman et al., 2015). Acetate and H_2 are some additional products which are used by the methanogens to produce methane (Khan et al., 2018) Eq. (3). Facultative/strict anaerobes such as *Enterobacter*, *Bacteriodes*, *streptococcus, Clostridium* spp. (*Clostridium cellulolyticum, Clostridium thermocellum*, *Clostridium cellulosi*), *Rumicoccus obeum*, *Acetivibrio* spp. (Niu et al., 2014, Azman et al., 2015) are known to facilitate the hydrolysis step of anaerobic digestion (Bajpai, 2017). The rate of hydrolysis can be limited by the type of substrate being utilised, particle size, pH, temperature, and production of enzymes (Ali Shah et al., 2014).

$$C_6H_{10}O_6 + 2H_2O \rightarrow -C_6H_{12}O_6 + H_2 \quad (3)$$

Hydrolysis is immediately followed by the acid–forming step known as acidogenesis (Khan et al., 2018). During acidognesis, the organics formed during hydrolysis are converted to higher organic acids such as propionic acid and butyric acid, in addition to acetic acid, H_2 and carbon dioxide by the acid-forming bacteria (Bundhoo, 2017). These organic acids (propionic acid and butyric acid) can be further broken down to acetic acid and H_2 by

acetogenic bacteria through a process called acetogenesis (Hansen and Cheong, 2013, Ali Shah et al., 2014). By–products acetate and H_2 are produced during both acidification and acetogenic steps. It difficult to differentiate these two steps in anaerobic digestion process (Adekunle and Okolie, 2015). Fermentative bacteria that facilitates these process are either facultative or strict anaerobes, such as *Enterobacteriaceae*, *Thermotoga maritima*, *Clostridium thermocellum*, *Escherichia coli*, *Bacillus cereus*, *Ruminococcus albus* and *Anaerococcus* (Lalman et al., 2013, Bajpai, 2017).

Acetogenic bacteria that carries this process include *Synthrophomonas wolfei*, *Smithella spp*. and *Synthophobacter wolinii*. The syntrophic acetogens (*Synthrophomonas wolfei* and *Synthophobacter wolinii*) carries out the oxidation of propionate and butyrate to acetate and H_2. Homoacetogens (*Smithella* spp.) also known as H_2 scavengers, are strictly anaerobes that uses CO_2/ H_2 as a substrate to produce acetate (Adekunle and Okolie, 2015) Eq. (4).

$$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2$$

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

$$CH_3CH_2OH + H_2O \rightarrow CH_3COO^- + 2H_2 + H^+ \text{ (Bajpai, 2017)}$$

$$4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4H_2O$$

(4)

The final stage is the methanogenesis carried out by methanogenic archaea (Bundhoo, 2017, Cisneros-Pérez et al., 2017) as shown in Figure 1. These archaea are capable of metabolizing formic acid, acetic acid, methanol, carbon monoxide, and carbon dioxide and H_2 to methane as shown in Eq. (5) (Ali Shah et al., 2014, Adekunle and Okolie, 2015).

$$CH_3COOH \rightarrow CH_4 + CO_2$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

$$2CH_3COCHOHCH_3 + 2CO_2 + 2H_2O \rightarrow 5CH_3COOH \quad (5)$$

The methanogenic microorganisms are slow growers and are vulnerable to harsh environmental conditions (Sivagurunathan et al., 2016a, Bajpai, 2017). Some of the known methanogenic archaea include *Methanobacterium bryantic*, *M. formicicum*, *M. thermoautotrophicum*, *Methanobrevibacter ruminantium* (Niu et al., 2014, Bajpai, 2017). metabolic processes of methanogenic archaea involve the consumption of hydrogen thus it is necessary to eliminate them in the system in order to produce high hydrogen yields (Rafieenia et al., 2018a). This step can be deactivated by inhibiting the metabolic growth of methanogens through enrichment methods (Jaseena and Sosamony, 2016).

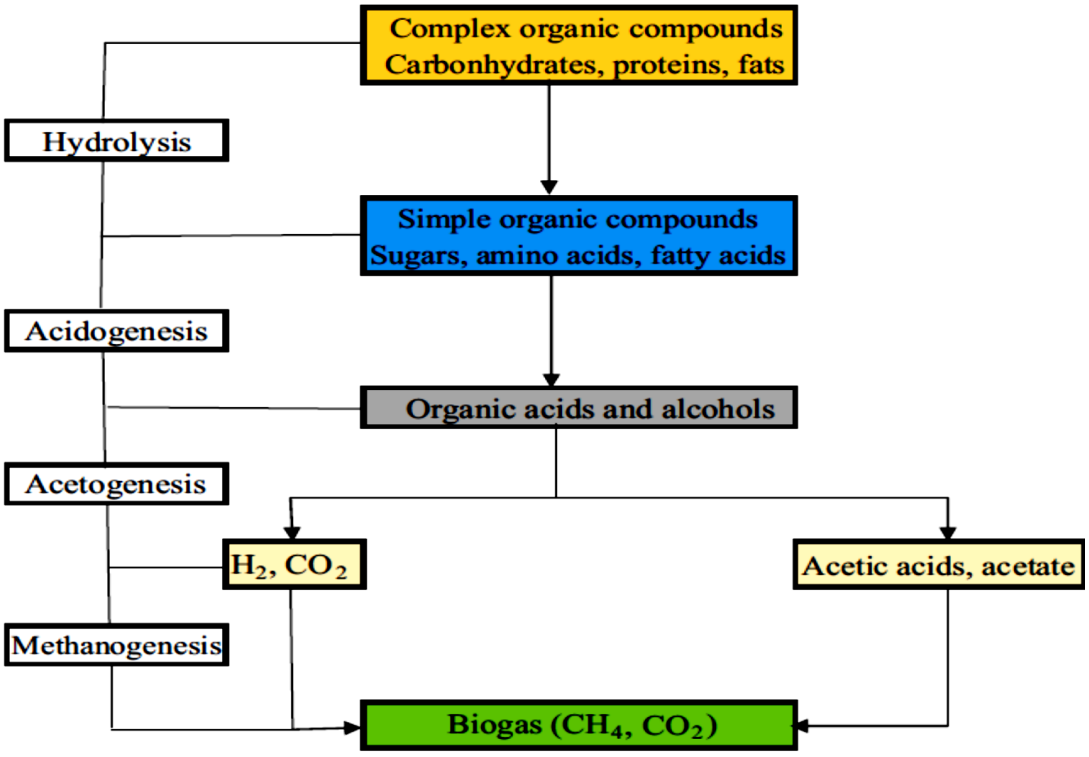


Figure 1. Biological fermentative breakdown of organic material (Matheri et al., 2017)

2.3.2 Factors affecting dark fermentation

Currently research on dark fermentative H_2 production has significantly improved but not yet fully reached the standards of being economically viable compared to conventional H_2 production methods (Sivagurunathan et al., 2016a, Argun et al., 2017). There are several factors that affect high productivity, rate and purity of H_2 production (Ghimire et al., 2015a, Sivagurunathan et al., 2016a). This includes pH, temperature, HRT as well as substrate and inoculum type.

2.3.2.1 pH

Hydrogen yield and rate in dark fermentation can be significantly influenced by the changes in pH as it affects the expression of enzymes that are responsible for different metabolic products (Chandrasekhar et al., 2015, Zagrodnik and Łaniecki, 2017). Control in pH play a crucial role in controlling and inhibiting the activity of H_2 consuming microorganisms. Acidic pH (4–6) favours the H_2 producing microorganisms such as *Clostridium* and the accumulation of the methanogens is favoured by alkaline conditions (Wang and Yin, 2017). However, very acidic, pH less than 4, could significantly inhibit the hydrogenase enzyme activity (Chuang et al., 2011). Previous studies reported an optimum pH for H_2 producers ranging from pH 5.5 to 6.5 (Sivagurunathan et al., 2016a, Vi et al., 2017) and for the methanogens from pH 6.0 to pH 7.5 (Chandrasekhar et al., 2015). Acetate production has been shown to be high at acidic pH 5–5.5 and butyrate production were produced at pH 5.5–6.5 (Waligórska, 2012). However, some reports argued that alkaline conditions result in acetate production and butyrate pathway is favoured by acidic conditions (Ghimire et al., 2015a). Operational factors such as temperatures, the type of substrates and substrate concentrations have been reported to influence the optimum pH (Ghimire et al., 2015a, Jaseena and Sosamony, 2016). Studies indicated that too much substrate concentration could

result in the accumulation of organic acids and lead to a decrease in pH thereby interfering with the hydrogenase activity (Lin et al., 2015).

2.3.2.2 Temperature

Temperature is one of the key factors affecting the microbial growth rate, substrate hydrolysis rate, metabolic products and consequently the overall H_2 production (Elsharnouby et al., 2013, Sivagurunathan et al., 2016a). Dark fermentation can be operated under mesophilic (35°C) (Ghimire et al., 2015a), thermophilic (55°C), extreme thermophilic temperature (60 – 65°C) and hyper thermophilic (70°C) (Ghimire et al., 2015a). Mesophilic temperatures are usually preferred due to low cost. A recent study obtained the highest H_2 yield of 2.42 mol H_2/mol mannose under mesophilic temperature of 35°C (Mishra et al., 2017b). Studies on lignocellulosic biomass as a substrate reported a requirement for high temperature conditions to achieve improved hydrolysis and high H_2 yields (Sivagurunathan et al., 2016a). Thermophilic temperatures can also be used to eliminate the methanogens or other unwanted microorganisms in the reactor (Jaseena and Sosamony, 2016). High H_2 production from wheat straw was obtained with extreme thermophilic conditions (Guo et al., 2010, Kongjan and Angelidaki, 2010). Bioreactor temperatures can also influence the metabolic pathways in the dark fermentation process, for example butyrate and acetate pathways are reported to favour high H_2 yield compared to ethanol and propionic at mesophilic conditions (Elbeshbishy et al., 2017). Temperature of 20°C was previously reported to lead to ethanol pathway and the accumulation of butyrate was observed at 37°C (Elbeshbishy et al., 2017). A recent study on mesophilic (35°C) biohydrogen production from xylose also observed butyrate as the major metabolic product (Qiu et al., 2016).

2.3.2.3 Hydraulic Retention Time (HRT)

Hydraulic retention time (HRT) affects hydrogen gas production during dark fermentation by influencing the rate of substrate degradation and production of metabolic products (Khanna and Das, 2013, Kirli and Karapinar, 2018). Hydrogen consuming microorganisms, a sub–group within methanogenic archaea reportedly have slow growth rate (0.0167 h^{-1} – 0.02 h^{-1}) as compared to the H_2 producers with a faster growth rate of 0.172 h^{-1} (Sivagurunathan et al., 2016a). Hydrogen production yields can therefore be improved by selecting for operational time based on the microbial population growth rate that could retain hydrogen producers while allowing washout of H_2 consumers. A previous study reported improved H_2 and inhibition of propionic inhibitor concentration when the HRT was reduced from 8 h to 6 h (Show et al., 2011). Another study obtained the highest H_2 yield and rate of 2.3 H_2 /mol glucose added and 78 L/H_2/L-d when the HRT was reduced from 12 h to 1.5 h, with butyrate and acetate as the main soluble metabolic products (Pugazhendhi et al., 2017b). Homoacetogens were also supressed when up flow anaerobic sludge blanket (UASB) and packed bed reactor (PBR) reactors were operated at 4 h compared to 24 h and the hydrogen consumption by methanogens was reduced from 12.1% to 3.1%. (Si et al., 2015b). A maximum H_2 yield of 1.47mol/mol glucose and H_2 production rate of 4.38L/L/d were observed at 8 h in UASB (Si et al., 2015a). H_2 production from acid hydrolysed wheat increased as the HRT was decreased (Kirli and Karapinar, 2018). HRT and pH was also concluded to have a joint effect on hydrogen production (Shi et al., 2017, Silva-Illanes et al., 2017) thus it is of significance to optimise both operational factors for enhanced H_2 yield and rate in dark fermentation.

2.3.2.4 Inoculum

Inoculum provides a start–up microbial culture for dark fermentative H_2 production. Studies have shown that H_2 producing consortium includes strict anaerobes (*Clostridia*) (Penniston and Gueguim Kana, 2018), facultative (*Enterobacter, E. coli, Citrobacter*) (Sun et al., 2015), thermoanerobacterium (*thermotogale, thermosacharolyticum, desulfotomaculum geothermicum*) as well as obligate aerobes (*Bacillus*) (Bundhoo et al., 2015, Laxman Pachapur et al., 2015, Hansen and Perner, 2016). The dark fermentation process can be conducted using either mixed culture, pure culture or combined pure culture (Chandrasekhar et al., 2015, Argun et al., 2017). Pure culture studies have been conducted for biohydrogen production utilising mostly *Clostridium* spp., *Enterobacteriaceae* (Mishra and Das, 2014), *Bacillus anthracis* (Mishra et al., 2017b) and *Bacillus cereus* (Sutthipattanasomboon and Wongthanate, 2017) each with different H_2 production yield. Therefore, fermentative condition of each strain should be optimized and controlled to enhance the H_2 productivity in the pure culture system.

Pure cultures are considered efficient in hydrogen production due to the absence of H_2–consuming and competing microorganisms as well as the ability to easily detect and control metabolic shifts (Antonopoulou et al., 2007, Elsharnouby et al., 2013, Łukajtis et al., 2018). However, pure cultures are limited by the requirement for expensive sterilization and energy consumption (Łukajtis et al., 2018). Co–cultures are defined as the combination of pure isolates with the aim to overcome the limitations in single pure culture and to enhance H_2 production (Laxman Pachapur et al., 2015). A previous study concluded that *Bacillus* spp. had a limited efficiency as a single pure culture which resulted in limited metabolic products and low H_2 yield compared to the co–culture of *Bacillus spp*. and *Brevumdimonas* spp. (Bao et al., 2012). Another study indicated a high H_2 yield of 3 mol/mol of glucose when a co–

culture of *Bacillus cereus*, *Enterobacter cloacae*, *Klebsiella* and *Citrobacter* were combined (Patel et al., 2014).

Mixed culture of facultative anaerobic and obligate anaerobic microorganisms have been studied by batch culture methods (Qian et al., 2011). Mixed cultures can be obtained from environmental sources such as sewage sludge, soil, anaerobic digester sludge, compost or manure (Gu et al., 2014, Ghimire et al., 2015a, Ghimire et al., 2015b). Recent studies have shown the potential of using anaerobic sludge as an inoculum due to the presence of high concentration of acidogenic and acetogenic microorganisms (Si et al., 2015a). They are cost effective, simpler to adjust and contains a wide variety of microorganisms (Chaitanya et al., 2018). A few studies indicated improved H_2 yield of mixed cultures compared to pure and combined pure cultures (de Sá et al., 2013, Wang and Yin, 2017). However, the challenge of using anaerobic mixed culture is the presence of H_2 consuming microorganisms, homoacetogens as well as sulfur reducing bacteria which all needs to be eliminated or inactivated for improved H_2 yields (Singh and Wahid, 2015). Several pre–treatment methods have been investigated to inactivate H_2 consumers considering that H_2 consumers are susceptible to harsh conditions such as acidic pH and thermal treatment (de Sá et al., 2011). H_2 producers tend to form spores during harsh conditions and germinate in favorable conditions (de Sá et al., 2011, Ghimire et al., 2015a, Elbeshbishy et al., 2017).

The co-culture of different microorganisms is found to be more advantageous over single culture due to the potential synergy between different H_2 producing microbes a resulting in increased HY (Cabrol et al., 2017b). In co–culture system, more than one isolates with different metabolic efficiency are combined together to enhance the hydrolysis rate of substrate and to improve the cumulative H_2 production. Studies indicated that the co–culture

promotes high H_2 yield at a very short fermentation time, offers a better process stability and a cheaper production process due to elimination of inoculum pre–treatment step (Elsharnouby et al., 2013, Pachapur et al., 2015, Sivagurunathan et al., 2016b). In some cases, having more than one species with different metabolic efficiency in the same system can also be detrimental as the species with lower H_2 producing capability can compete with the species with higher producing ability for the substrate leading to an overall reduction in hydrogen yields. Therefore, selection of potential candidates plays a crucial role in the success and failure of a system. Few studies however, have shown that the co–cultures are more advantageous over the mixed culture and monoculture due to the synergetic effect offered by the combined pure cultures (Elsharnouby et al., 2013, Mu et al., 2014, Pachapur et al., 2015).

2.4 Sludge pre–treatment methods

There are several processes of sludge pre–treatment classified as physical or chemical.

2.4.1 Physical pre–treatment

Physical methods of sludge pre–treatment includes heat–shock, ultra–sonication, microwave irradiation, freezing and thawing, and aeration (Rafieenia et al., 2018a). The thermal or heat–shock pretreatment is one of the most employed pre–treatment for mixed anaerobic inoculum because it is simple, cheap and effective (Ennouri et al., 2016). Heat–shock is carried out by boiling, autoclaving or drying the sludge (Sivagurunathan et al., 2016a). The heat treatment at 100°C–121°C inactivate/kill non–spore forming microorganisms while enriching the spore forming bacteria such as *Clostridium* spp. and *Bacillus* spp. (Wang and Yin, 2017). Different temperatures and time have been used for the heat–shock treatment, ranging between 65°C –121°C for short retention times of 15 minutes to 2h (Sivagurunathan et al., 2016a). Freezing and thawing pre–treatment method is carried out in low temperature

conditions between –25°C to –10°C followed by heating at 30°C, however, it has been reported to be partially effective (Rafieenia et al., 2018a). Aeration treatment allows exposure of the inoculum to air (oxygen) which slows down the growth activity of unwanted microbes since they are mostly strict anaerobes and very sensitive to oxygen (Giordano et al., 2014). The spore forming microorganisms re–germinate when the conditions are shifted back to anaerobic.

2.4.2 Chemical pre–treatment

Chemical methods include the use of acid or bases as well as growth inhibitors for sludge pre–treatment to eliminate the H_2 consumers (Salem et al., 2018). High H_2 yields can be achieved by adjusting pH to a level that inhibits hydrogen consuming bacteria while enriching hydrogen producing microorganisms. The principle is that very acidic pH of up to 4.5 (Sivagurunathan et al., 2016a) and above 7.5 inhibits and slows down the growth of methanogens while *Clostridium* species are enriched (Jaseena and Sosamony, 2016). Another study reported growth inhibition of methanogens at acidic medium with pH in the range of 2–4 and for an alkaline medium it varies from 10 to 12 (Sunyoto et al., 2017). Chemicals such as acetylene, 2–bromoethanesulfonate, 2–bromosulfonic acid and chloroform, iodo–propane, linoleic acid acts as a growth inhibitor for hydrogen consumers (Rafieenia et al., 2018a). A previous study completely eliminated the activity of methanogens in anaerobic sludge when 10 mmol of 2–bromoethanesulfonic acid was used for the pre–treatment of the inoculum at room temperature (Łukajtis et al., 2018). Improved dark fermentative hydrogen production was also achieved when chloroform was used for the pre–treatment of the inoculum (Ning et al., 2012).

2.4.3 Substrate to biomass (S/X) ratio

Optimization and adjustment of initial food to microorganism ratio is important in dark fermentation for controlled process conditions as well as in designing the reactor (Barua and Kalamdhad, 2018). High substrate concentration has been reported to lead to increased H_2 yield (Akutsu et al., 2009, Lu et al., 2018). While other studies concluded that accumulation of metabolic products due to high substrate to biomass lead to reduction in pH and H_2 yield (Wongthanate and Chinnacotpong, 2015, Barua and Kalamdhad, 2018). Balanced substrate to biomass ratio has been reported to be influenced by temperatures (Pan et al., 2008). Previously a better H_2 yield of 57 ml/g VS and volatile solid reduction (39%) was obtained at optimum S/X 7 under thermophilic temperatures compared to mesophilic temperatures with 39 ml/g VS and 46% volatile reduction at S/X 6 (Pan et al., 2008). It was also observed that when the S/X was increased from 1–5 in thermophilic conditions, it resulted in enhanced H_2 yield and reduced CH_4 compared to mesophilic conditions (Pan et al., 2008). Anaerobic biodegradability test using *Eichhornia crassipes* as a substrate observed a balanced substrate to biomass ratio of 1.5 (Barua and Kalamdhad, 2018). A recent study investigated the effect of different substrate to biomass ratio in mesophilic dark fermentation and observed 0.07 optimum S/X ratio compared to 0.01 and 0.14 dry weight basis (Florio et al., 2017). However, too much of substrate can also results in substrate/product inhibition and thus low H_2 yield (Nissilä et al., 2014, Argun and Dao, 2016, Sivagurunathan et al., 2016a). Studies also investigated the interaction of the operational factors affecting dark fermentation (pH, initial substrate and biomass) and concluded that S/X had less significant effect on H_2 yield while pH was found to have a major effect on H_2 yield (Tunçay et al., 2017).

2.4.4 Substrate

The substrate for dark fermentative H_2 production must be highly biodegradable, readily available and have a high carbohydrates content (Ghimire et al., 2016). Moreover, the costs of raw material can be a challenge in biological H_2 production (Pandu and Joseph, 2012). Different types of substrate are utilised for dark fermentation viz. pure glucose and xylose are ideal substrates, they result in high H_2 yield and rate however they are expensive to utilise (Subudhi et al., 2013). First generation substrates for biohydrogen recently gained attention due to rich–sugar content and low lignin content, they are directly attained from food crops such as corn (García et al., 2017), vegetable oil, potato or wheat (Vi et al., 2017, Kirli and Karapinar, 2018) and wastewater effluent from municipal or industrial such as palm oil mill effluent (Nitipan et al., 2014, Mamimin et al., 2017). The second generation substrate for H_2 production consist of the lignocellulosic materials such as sugar cane bagasse (Reddy et al., 2017), aquatic weeds (Miranda et al., 2016b, Corneli et al., 2017), rice (Chaudhary et al., 2015), corn stalk, reed straw (Hu et al., 2013) and wheat straw (Shi et al., 2017). Utilisation of waste material such as aquatic weeds can be beneficial as a resource recovery and in cleaning up the pollution in the environment. Lignocellulosic waste biomass are however more advantageous for H_2 production since they do not compromise food security, or compete with agricultural land (Kapdan and Kargi, 2006). Additionally, lignocellulosic waste biomass is considered a potential substrate with adequate carbohydrate content to reduce the costs of energy production (Kapdan and Kargi, 2006).

2.4.5 Pre–treatment of lignocellulosic biomass

The main limitation of utilising aquatic weeds for biofuel production is the complex structure of the lignocellulosic material and its high moisture content (Barakat et al., 2015, Argun et al., 2017). It is composed of covalent cross–linkages joining cellulose, hemicellulose and

lignin (Figure 2) (Table 2) (Amin et al., 2016, Amin et al., 2017). Cellulose fibre gives rise to simple sugar monomers such as glucose while hemicelluloses consist of xylose and arabinose and inter–connect lignin and the cellulose (Barakat et al., 2015). Lignin is the protective cell wall of the plant material, it is the main factor that affect hydrolysis of the plant biomass and mainly consist of fermentation inhibitors such as furfural, phenolic compounds and 5-(Hydroxymethyl) furfural (5–HMF) (Barakat et al., 2015). Pre–treatment of the lignocellulosic material using mechanical, chemical and biological or combined methods are very important in breaking down the complex structure to facilitate easy digestion and fast hydrolysis by the microorganisms (Harun et al., 2011, Amin et al., 2016, Amin et al., 2017).

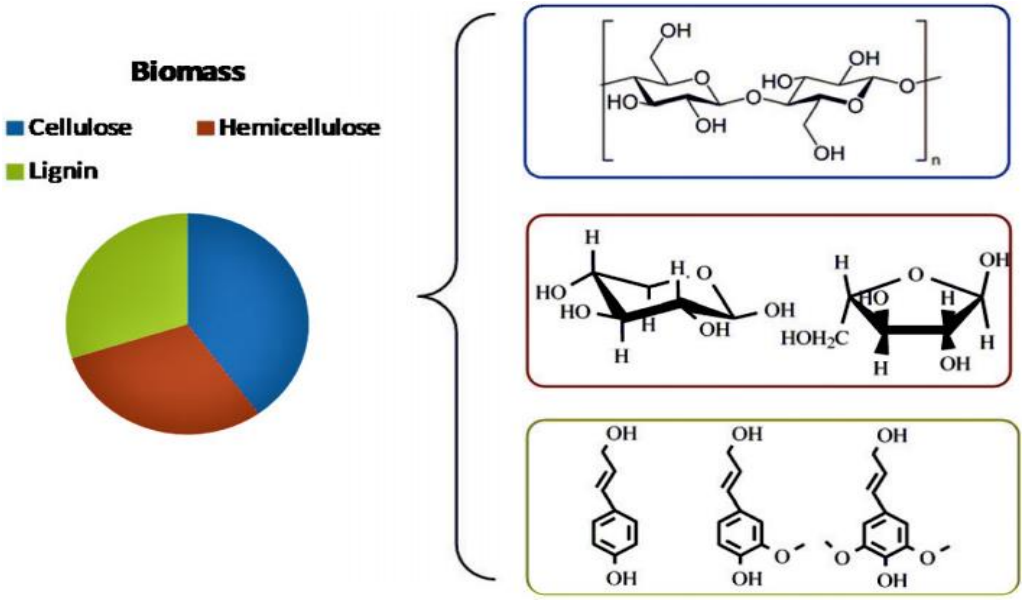


Figure 2. Composition of lignocellulosic biomass (Wang et al., 2014)

a) Physical pre-treatment

Physical treatment (milling, chipping/grinding and sonication) is the mechanical breakdown of lignin and dissociation of the biomass with the aim of decreasing crystallinity and

intensity of polymerization of hemicellulose and cellulose leading to increased surface area and pore sizes of the biomass (Barakat et al., 2014, Barakat et al., 2015). This treatment is mostly explored since, it does not require chemicals or water addition, thus cost effective, but results in low sugar recovery (Barakat et al., 2014).

b) Chemical pre–treatment

Chemical treatment is the use of strong acid and bases to delignify the lignin and reduce crystallinity of cellulose (Łukajtis et al., 2018). Sulphuric acid (H_2SO_4), acetic acid (CH_3COOH), hydrochloric acid (HCL), sodium hydroxide (NaOH), lime ($Ca(OH_2$) and H_2 peroxide (H_2O_2) are some of the reported acids and bases employed (Amin et al., 2017). This process is efficient and mostly used but the limitation is the high cost of chemicals needed additionally, high concentration of acid causes corrosion of equipment, is toxic to protective clothing and require reactors that are resistant to corrosion (Bundhoo et al., 2015). Thus, after the completion of acid hydrolysis, the pH must be regulated to neutral values to avoid even further corrosion. Alkali methods such as the use of lime and ammonia is cost–effective but requires longer incubation time for improved penetration therefore, this process is more efficient in biomass with lower lignin content (Amin et al., 2017).

c) Physicochemical pre–treatment

Physicochemical processes involve combination of acids, steam explosion, boiling and microwave treatments (Kim, 2018). Steam explosion is often used due to its high productivity, it completely breaks down the lignin and separate the tissues leading to complete sugar recovery by use of high pressure saturated steam and temperature (Hu et al., 2013, Jacquet et al., 2015, Kim, 2018). This process has low hazardous environmental impact however they can result in partial conversion of released sugars to toxic compounds

such as phenolics and furfurals (Harun et al., 2011, Kim, 2018). Boiling solubilize mainly the hemicellulose, make the cellulose more accessible and avoid formation of inhibitors, however, if it is not properly optimized it can result in partial delignification with high energy consumption (Reddy, 2016). The microwave radiation involves application of forces by electric and magnetic field to dissociate or disintegrate the complex structure (Chen et al., 2017). It is mostly applied to speed up processes such as chemical, biological and physical (Amin et al., 2017). Although, combination of the pre–treatment methods enhance complete sugar recovery (Huang et al., 2017), they also increase the cost of production. Studies on the combination of pre-treatment methods are still ongoing to reduce the costs and develop a sustainable efficient pre–treatment method.

d) Biological pre–treatment

Biological pre–treatments are also used to destroy the lignocellulose structure and they include enzymatic, bacterial, and fungal treatment (Rafieenia et al., 2018a). Fungal treatment employ specific microorganisms to degrade lignin, hemicellulose and cellulose such as soft–rot fungi, brown–rot (*Antrodia*) and white –rot fungus (*E. taxodii*) (Myat and Ryu, 2016). Fungus *Trichoderma reesei Rut C-30, Actinomycetes* and *Phanerochaete chrysosporium* have been use in fungal treatment (Reddy, 2016). Enzymatic hydrolysis employ direct of enzymes arabinase, xylase, cellulose, hemicellulase, and b-glucanase to degrade lignin structure (Rabemanolontsoa and Saka, 2016). The biological process is less desirable due to the slow hydrolysis process (long residence time), the use of pure culture which requires strict conditions and some hydrolysis microbes can utilise the released sugars (Chen et al., 2017) (Figure 3).

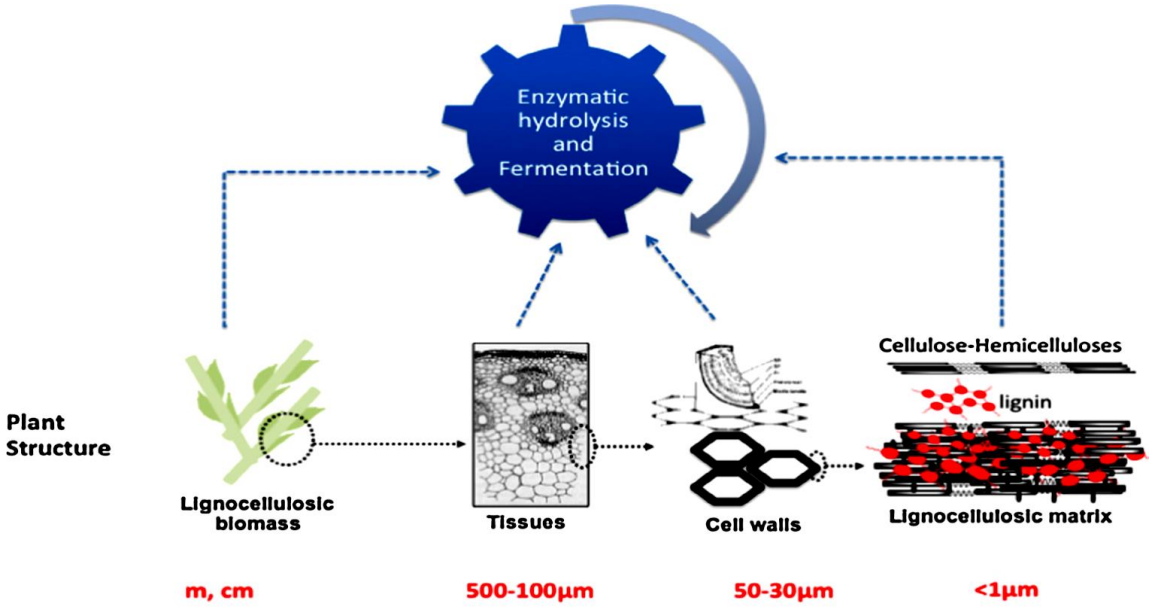


Figure 3. Lignocellulose breakdown by enzymatic pre–treatment method (Barakat et al., 2015)

2.6 *Pistia stratiotes* (Water lettuce)

Invasion of surface waters by aquatic weeds is one of the major issues facing nations due to their adaptability to varying environmental conditions (Coetzee and Hill, 2011, Gusain and Suthar, 2017). These invasive plants rapidly spread across water bodies disturbing water supply, hydroelectric functioning, poses health risk to both wildlife and humans and leads to loss of water through transpiration (Chamier et al., 2012).

Pistia stratiotes commonly known as water lettuce, is one of the worst aquatic weeds in South Africa. It originated as ornamental plant and is well known for its reproductive capacity, high moisture content and its bioremediation abilities (Rezania et al., 2015). In South Africa, more than ZAR350 million has been used on control and management between year 1997 and year 2016 alone (Fraser et al., 2016, van Wilgen et al., 2017). This free floating aquatic weed along with water hyacinth, Kariba weed, water velvet (Harun et al.,

2011, Chamier et al., 2012, Gusain and Suthar, 2017) is currently spreading at an alarming rate causing a big threat to South Africa's water security and diversity. Areas that have been infested in South Africa include North–eastern (Kruger national park) (van Wilgen et al., 2017), KwaZulu–Natal (Inanda dam, Umngeni catchment, Umngeni river, Hammarsdale) and Cape town (Wemmershoek dam, Diep river of Bloubergrand ridge, Banana beach estuary) (May and Coetzee, 2013, Shonalanga, 2014).

These aquatic weeds are difficult to control, due to high growth rate, which doubles within two weeks, and has high moisture content (Coetzee and Hill, 2011). Physical, chemical and biological control methods have been employed to manage them however, these are either expensive or environmentally challenging (Malik, 2007). Eradication and management of established plants has been the biggest challenge due to negative externalities (reduced native, establishment of secondary invaders), costs and residual impacts (Panetta and Gooden, 2017). Studies have indicated the possibility of using the biomass of aquatic weeds (Table 2), to produce value added products such as biofuels (methane, hydrogen, ethanol etc.) (Chuang et al., 2011, Kaur et al., 2018, Varanasi et al., 2018). Therefore, utilising aquatic weeds as a renewable feedstock for bioenergy can be an efficient management method for cleaner energy production such as hydrogen and will ensure environmental protection, and also add research drive to replace non–renewable fossil fuels (Rickard *et al*., 2016).

Table 2. Chemical composition of aquatic weeds

Aquatic weeds	Carbohydrate content (%)	C: N Ratio	Reference
Pistia stratiotes (Water lettuce)	49.4	–	(Pantawong et al., 2015)
Azolla filiculoides (water fern)	41	15	(Miranda et al., 2016b)
Lemnoideae (Duckweeds)	20.3 (Glucan)	–	(Ge et al., 2012)
Lemnoideae (Duckweeds)	38	–	(Cui and Cheng, 2015)
Eichhornia crassipes (Water hyacinth)	40.7	29	(Mathew et al., 2015)
Salvinia (Watermoss)	32.2	23.0	(Mathew et al., 2015)
Pistia stratiotes (Water lettuce)	–	25.7	This study

2.7 Previous studies for fermentative biogas production from aquatic weeds

The use of aquatic weeds for biofuel production (Table 3) has caught a lot of attention recently due to its high productivity and cost effectiveness. Aquatic weed grows in water bodies and they do not compete for arable land therefore, they are considered not a threat to food security (Kaur et al., 2018). Several previous studies focused on biogas (methane and CO_2) and bioethanol production from aquatic weeds (Gaur et al., 2017, Gusain and Suthar,

2017, Sarto et al., 2019). *Eichhornia crassipes* (water hyacinth) is one of the mostly investigated aquatic weed for hydrogen production (Lay et al., 2013, Anaerob et al., 2017, Song et al., 2017). Aquatic weed, *P. stratiotes* has been utilised for bioethanol and methane production (Namadi M et al., 2013, Pantawong et al., 2015, Gusain and Suthar, 2017). Prior to this study, only one recent work has been published on biohydrogen production from *P. stratiotes* (via a photo fermentation pathway) (Corneli et al., 2017).

Table 3. Biofuel production from different aquatic weeds

Biomass (aquatic weeds/plants)	Biofuel yield (hydrogen, ethanol and methane(CH_4))	Reference
Azolla filiculoides	2.2 mol/mol glucose/xylose of hydrogen	(Miranda et al., 2016a)
Acid (H_2SO_4) treated–*Azolla filiculoides*	2.4 mol/substrate	(Miranda et al., 2016a)
Pistia stratiotes (Water lettuce)	(7.3 mL L^{-1} h^{-1}) and (5.6 mL L^{-1} h^{-1})	(Corneli et al., 2017)
Eichhornia crassipes (Water hyacinth)	17 ml H_2 g^{-1}	(Chuang et al., 2011)
Eichhornia crassipes (Water hyacinth)	76.7 mlH_2/20g/L water hyacinth	(Su et al., 2010)
Pistia stratiotes (Water lettuce)	66.35% CH_4 9667.33 ml/ total gas yield	(Pantawong et al., 2015)
Water hyacinth	62% CH_4	(Mathew et al., 2015)
Salvinia	63% CH_4	(Mathew et al., 2015)
Duck weed (*Lemnaceae)*	75 ml H_2/g-7days (42% H_2)	(Xu and Deshusses, 2015)
Duckweed	6.42x$10^3$$ha^{-1}$bioethanol (50% of that of maize)	(Xu et al., 2011)
Lemna minor	0.22g bioethanol/g^{-1} dry biomass	(Gusain and Suthar, 2017)
Lemna gibba	0.19g bioethanol/g^{-1} dry biomass	(Gusain and Suthar, 2017)
Pistia stratiotes (water lettuce)	0.22g bioethanol/g^{-1} dry biomass	(Gusain and Suthar, 2017)
Eichhornia crassipes (water hyacinth)	0.2g bioethanol/g^{-1} dry biomass	(Gusain and Suthar, 2017)

2.8 Techniques used to identify microbial communities in hydrogen production

The essential step towards understanding the function of different bacteria in both inoculum and hydrogen production bioreactor, is to detect and quantify the relevant bacteria responsible for the metabolic (Li et al., 2011) reactions.

Culture–dependent and non–culture dependent methods have been used to identify hydrogen producing microorganism. Culturable methods identify microorganism by means of characterization techniques and isolation in appropriate media (Wang et al., 2007). However, the limitation with culture–dependent techniques is that some cultures, such as environmental bacteria are unculturable (Pham and Kim, 2012). Culture independent techniques uses molecular techniques such as Fluorescent in situ hybridization (FISH), Polymerase Chain Reaction (PCR), PCR–Denaturing Gradient Gel Electrophoresis (DGGE), real–time PCR, as well as droplet digital PCR (Kumar et al., 2018a).

2.8.1 FISH

Fluorescent in-situ hybridization (FISH) is a visualisation technique used to characterize the inoculum and analyse the microbial community structure of hydrogen producers (Hwang et al., 2009a, Lee et al., 2009). It is based on microscopic examination after staining cells with fluorescent labelled and specific 16S rRNA oligonucleotides probes (Gorham et al., 2016). Laser or epifluorescence microscope are used for visualisation (Hwang et al., 2009. The specificity of the oligonucleotide probes allows detection from domain level to species level (Gorham et al., 2016). FISH does not require DNA extraction step which makes it an excellent detection method compared to other molecular based method such as PCR or real–time PCR (Kumar et al., 2018a). It allows visualisation of non-culturable bacteria, however, the identification requires evenly distributed cells thus allows semi-quantification (Hannig et al., 2010). The challenge of using FISH is the auto fluorescence background in the

environmental samples and flocs which affect analysis of images or cells as well as quantification (Mota et al., 2012). Pre–treatment methods have been used to break the bacterial floc for accurate enumeration. However, the challenge has been to select a procedure that is not harsh to cause cell disruption but able to achieve complete detachment of bacteria from flocculated clumps in environmental samples (Legge, 2012). The probes used in FISH are typically RNA-based and the RNA is often present in small quantity and degrades easily and if a new probe is needed for a target microorganism, RNA gene sequence must be known, moreover, it is challenging to optimise newly designed probe (Kumar et al., 2018). FISH have been successfully used in combination with other detection techniques such as denaturing gradient gel electrophoresis (DGGE) to overcome these limitations (Mishra et al., 2015). FISH has been applied to characterise microorganisms in the hydrogen-producing sludge before and after pre–treatments for seed culture in dark fermentation bioreactor (Reddy et al., 2017). The FISH technique has also been used with 16S rRNA-targeted oligonucleotide probes to monitor the change in the microbial community (Hwang et al., 2009a, Lee et al., 2009). Dominance of *Bacillus* species were monitored using FISH in biohydrogen production from starch process wastewater (Sutthipattanasomboon and Wongthanate, 2017).

2.8.2 Polymerase Chain Reaction (PCR) and Denaturing Gradient Gel Electrophoresis (DGGE)

PCR is a molecular technique that achieves amplification of specific region of DNA in various sample source (Rosselli et al., 2016). Universal and individual or domain targeted primers for 16S rRNA genes has been used to obtain PCR amplicons from extracted metagenomic DNA (Lorenz, 2012). The difference in microbial community is based on the visualisation of band patterns in a gel electrophoresis. The draw backs in PCR is the presence

of inhibitors in metagenomics samples, it is time consuming and not quantitative. DGGE is a PCR based technique, it is also a simple and rapid monitoring method of target microorganisms during the biological processes (Mota et al., 2012). DGGE is performed by taking PCR results in DNA segments with different sequences but same size, the segments are then separated in an acrylamide gel, but not in an agarose gel, having a linear ascending gradient of denaturants, usually urea and formamide (Carmona et al., 2011). DGGE technique has been widely used in monitoring and estimating the abundance of microbial species (Piterina and Pembroke, 2013). *Clostridium* spp. were identified from activated sludge using 16S rRNA gene-directed polymerase chain reaction-DGGE (Wang et al., 2007). Both PCR and DGGE are cost effective for large quantity of samples. Previous studies have successfully used PCR for detection and monitoring of both hydrogen producers and hydrogen consuming microorganisms (Cao et al., 2014, Reddy et al., 2017, Kumar et al., 2018a).

2.8.3 quantitative PCR

The qPCR is one of the advanced techniques mostly used for identification and quantification of microorganisms in real time. The qPCR has better sensitivity or precision and capable of detecting DNA and RNA at low concentrations compared to conventional PCR methods (Tolvanen et al., 2008, Pugazhendhi et al., 2017a). It is considered time consuming since it evaluates microorganism in real time. Detection or precision in qPCR is however, limited by the depletion of components in the reaction as the number of cycle increases (Kumar et al., 2018a). Detection techniques such as 16SrDNA based tools have contributed a lot of knowledge in the development of hydrogen production studies, such as exploration of microbial ecology of hydrogen producing consortia (Pugazhendhi et al., 2017a, Banu et al., 2018). Research is still on going to advance and further develop

molecular based methods aimed at improving and understanding hydrogen production performance in bioreactors. Next Generation sequencing (NGS), Amplicon sequencing or phylogenetic identification are one of the most advanced methods for monitoring microbial community. These techniques offers a complete view of genetic diversity compared to other molecular techniques that only targets single or few genes.

2.8.4 Sequencing

DNA sequencing techniques can be used to identify and quantify abundant microbial community and provides information on phylogenetic and functional interactions (Kumar et al., 2018). Sequencing techniques analyse the whole genetic information present in DNA extracted from mixed culture or pure culture (Legge, 2012). Next Generation sequencing (NGS) is a high throughput method that has been used in dark fermentation studies to analyse the entire population and understand key microbial elements driving the processes which cannot be analysed by standard methods (Antony et al., 2016). NGS allows sequencing of huge amount of DNA in a short period of time and can be analysed using bioinformatics tools and experts to interpret the obtained data (Rucha et al 2018). NGS includes illumina sequencing, Roche 454 sequencing, ion Torrent and solid sequencing (Ambardar et al., 2016). Hydrogen producing microbial community can be monitored by 16S rRNA gene sequencing (amplicon sequencing) and metagenomic whole genome sequencing. Shotgun sequencing reveal phylogeny and microbial function present in environmental sample (Fantini et al., 2015, Salipante et al., 2014). However, the 16S rRNA gene will not provide data on the function of the organisms in the environment if the identified microbe is new and not well classified (Poretsky et al.,2014). Amplicon sequencing provides a very sensitive tool for identification and quantification of low DNA concentration based on the taxonomy

present in the sample (Kumar et al., 2018). NGS offers a very cost effective and thorough classification technique for monitoring microbial community.

CHAPTER I I I

OPTIMIZATION OF PRE-TREATMENT METHODS TO HYDROLYSE THE BIOMASS OF *PISTIA STRATIOTES* AND CHARACTERIZATION OF THE HYDROLYSATE

3.1 Introduction

Aquatic weeds are one of the most abundant and readily accessible biomasses with a high carbohydrate content (Miranda et al., 2016a, Gusain and Suthar, 2017). They are complex lignocellulosic structure mainly containing lignin, hemicellulose and cellulose (Rismiller et al., 2018). In search for a sustainable energy source, the aquatic biomass can be a potential renewable feedstock for H_2 production. However, its suitability for adequate H_2 production greatly depends on its C: N ratio and biodegradability (Usmani et al., 2017). Additionally, the recalcitrant structure of lignocellulosic biomass hinders the release of readily fermentable sugars which causes hydrolysis to become a rate limiting step in dark fermentation (Haldar et al., 2016, Myat and Ryu, 2016, Sun et al., 2016, Paudel et al., 2017). It is therefore imperative to hydrolyse the plant biomass prior to fermentation to enhance the availability of cellulose to enzymes and degrade the carbohydrates into reducing sugars.

The pre–treatment method selected for hydrolysis of the substrate should be able to provide high sugar recovery within a minimal period, at an affordable cost and without any inhibitor concentration (Haldar et al., 2016). Several methods have been employed for the pre–treatment of complex lignocellulosic biomass, these methods are explained in complete details in chapter II (section 2.5.7(a) to (d).

Characterization of the substrate provides a better understanding of pre–treatment requirements of the biomass as well as its digestibility. Therefore, this objective focused on characterization of the *P. stratiotes* biomass. The substrate was subjected to different pre–treatment methods i.e. physical, chemical and physical–chemical methods to improve its susceptibility to hydrolysis and for production of maximum fermentable sugars. The morphological changes before and after the pre–treatment were examined for correlation between the morphological disruption and the optimum pre–treatment method.

3.2 Materials and methods

3.2.1 Substrate preparation

Fresh water lettuce (*P. stratiotes*) was harvested manually from a river in Durban, KwaZulu–Natal. The harvested biomass was washed with tap water, the roots and rotten parts were discarded since they are reported to contain heavy metal that can affect dark fermentation results (Harun et al., 2011). The sample in this study was therefore refered as raw sample soon after sampling and cleaning. The remaining parts of the biomass were dried at 105°C for 24 h using an oven (Labcon, South Africa) (Namadi M et al., 2013). The dried plant biomass was then milled using a stainless steel grinder (Waring Laboratory Science, South Africa) and sieved (0.5mm pore size) to obtain a particle size between 0.25 mm and 0.5 mm. The filtered samples were then stored in plastic bags for 1-2 weeks (room temperature) while doing the analysis, and pre–treatments. The structure of the lignocellulosic requires subjection to various enzymes (mainly cellulases and hemicellulases) and pre–treatment methods (dilute acid, steam explosion, alkaline peroxide) to be degraded. Short storage at room temperature do not easily degrade essential components required for dark fermentation (Pattra and Sittijunda, 2015).

3.2.2 Characterization

The dried plant sample were analysed for ash, total solids, moisture content, and C: N using a DR6000™ UV VIS Spectrophotometer (HACH, RFID Technology), furnace, and oven (Lubicon, South Africa), following Saviour and Nielsen (2010). The cellulose, hemicellulose and lignin components of *P. stratiotes* were determined by HPLC (Shimadzu, Japan) with a refractive index detector and Aminex HPX-87H column (300 × 7.8 mm) at 40°C, using the Gravimetric analysis (strong acid hydrolysis) from National Renewable

Energy Laboratory (NREL) method (Sluiter et al., 2011), Hemicellulose and cellulose was then calculated as stated below-

- Cellulose%= Glucose/1.11(conversion factor for glucose based polymers) *100
- Hemicellulose%= Xylose + Arabinose/1.13 (conversion factor for xylose based polymers to monomers)

Acid –soluble and acid-insoluble lignin was determined using the method "Determination of structural and lignin in biomass" by NREL methods (Sluiter et al., 2011) as discussed below-

(a) Determination of insoluble lignin

The insoluble material retained on the filter paper after hydrolysis and filtration of the hydrolysate) was washed with about 1.5 L of distilled water to remove residual acid (until pH near 7) and dry in oven at 105°C until constant mass. The percentage of insoluble lignin was calculated in relation to the mass of dry lignocellulosic material discounting the mass of ashes (inorganic compounds).

(b) Determination of acid soluble lignin

The amount of soluble lignin was determined by measuring absorbance at 280 nm in a UV-visible spectrophotometer. Background of deionized water was run first as a blank and sing the hydrolysis liquor aliquot obtained in hydrolysis, the absorbance of the sample was measured. This analysis was done within six hours of hydrolysis. Acid soluble lignin (ASL) was determined using Eq. (6). below

$$\text{ASL} = \frac{UV absorbance\ X\ Vol\ of\ filtrate\ X\ dilution}{\text{Ɛ}\ X\ sample\ weight X\ Pathlenth}\ X\ 100$$

(6)

The substrate analysis (ash, total solids, moisture content, and C: N) are discussed below.

3.2.2.1 Determination of moisture content

To determine the moisture content, 3 g of properly mixed and evenly spread raw sample (*P. stratiotes*) was transferred to a weighing dish balance to record the initial weight. This sample was then dried in an oven at 105°C for 24 h and recorded the dry weight. This procedure was repeated until a constant dried weight was obtained. The difference in weight from initial to final was used to determine the total moisture content (LAP, 1998). The percentage of moisture content was determined using Eq. (7). The analysis was carried out in duplicate.

$$\%Moisture\ = \left(\frac{Initial\ weight\ plus\ dish\ -\ dried\ weight\ plus\ dish}{Initial\ weight\ plus\ dish}\right) X\ 100$$

(7)

3.2.2.2 Determination of total solids in biomass

Total solids in biomass was determined by first placing marked crucibles in a furnace at 575 ± 250°C to ignite all previous materials present in the crucible. The crucibles were removed and cooled down to room temperature in a desiccator and recorded the weight. Three gram of raw sample (*P. stratiotes*) was weighed, transferred into the crucible and recorded the weight of sample plus crucible. The crucible with sample was thereafter placed in a conventional oven at 105 ± 3°C for 4 hours. The crucible plus sample were then removed from the oven, allowed to cool down in a desiccator and the weight recorded. This was

repeated until a constant weight was obtained (LAP, 1998). The percentage of total solids was then determined according to Eq. (8).

$$\%Total\ solids\ = \left(\frac{Initial\ weight\ plus\ dish\ -\ dried\ weight\ plus\ dish}{Initial\ weight\ plus\ dish} \right) X\ 100 \quad (8)$$

3.2.2.3 Determination of Ash content

Ash content of the dry biomass was determined as described by Sluiter et al., (2011). Marked crucible were first ignited to remove previous materials present on the crucible as described in 3.2.2.2 (Sluiter et al., 2011). Three gram of 105°C dried sample was weight, transferred into the tared porceline crucible and both weight recorded. The crucible with sample was placed into a furnace at 550 ± 25°C for 3 hours. The samples were then removed and allowed to be cooled down to room temperature in a desiccator. The ignition step was repeated until a constant weight was retained. The final weight of the ash was recorded and the ash percentage calculated based on Eq. (9).

$$\%Ash\ = \left(\frac{Weight\ of\ ash\ plus\ dish\ -weight\ of\ dish}{weight\ of\ 105°C\ \ dried\ sample} \right) X\ 100 \quad (9)$$

3.2.2.4 C: N ratio

The C: N ratio was calculated using the total organic carbon (TOC) and the total Kjeldhal nitrogen (Sivasankari and David Ravindran, 2016, Divyabharathi et al., 2017). Total OC was determined by following the method described below (Divyabharathi et al., 2017).

Estimation of Total Organic Carbon (TOC)

The TOC was estimated following the wet digestion method as described by Divyabharathi (2017). The 20 ml sample was digested with 50-75 ml of 1 N $K_2Cr_2O_7$ with 20 ml of

concentration of H_2SO_4 using a heating block digester (Milestone S.R.L.). After 30 minutes 10 ml of Ortho phosphoric acid was added. This was titrated against 1 N Ferrous Ammonium Sulphate (FAS) with diphenylamine as indicator and the total organic carbon was calculated using Eq .(10).

$$\text{TOC(mg/L)} = \frac{Bv - Sv \ X \ NSFAS \ X \ 100 \ \ X \ 0.03}{Vs} \tag{10}$$

Bv is the Blank titre value, Sv is the Sample titre value, NFAS is the Normality of FAS and Vs is the Volume of test Sample

Total Kjeldhal nitrogen was determined by following the Nessler method described for DR6000™ UV VIS Spectrophotometer (HACH, RFID Technology). Two mL of 25% $KMnO_4$ solution was added to one mL (1000-56000 mg/L) powdered sample (*P. stratiotes*) followed by 3 drops of concentrated H_2SO_4. Approximately,10 mL of diacid (H_2SO_4 and $HCLO_3$) was then added to the reaction mixture and digested. After digestion 20 mL of 40% NaOH was added to 5 mL of the digested sample to distill and titrated against 0.05 N H_2SO_4. The digested samples were then analyzed using the DR 6000 instrument (Divyabharathi et al., 2017). The total Kjeldhal nitrogen was then calculated using Eq. 10. Then the C: N ratio was calculated by dividing the OC Eq. (10) by Eq. (11).

$$\text{TKN(mg/L)} = \frac{titre \ value \ X \ 14 \ X \ vol.acid \ made \ up}{vol.acid \ added} \ X \ 100 \tag{11}$$

3.2.3 Thermal treatment

To study the effect of thermal pre–treatment on the hydrolysis of *P. stratiotes*, the experiments were carried out under both boiling (100°C) temperature and steaming (121°C). The boiling pre-treatment experiment was conducted in a water bath at a temperature of 100 ± 3°C and steaming in an autoclave at 121°C. All the treatments were conducted by using 1g dried and powdered *P. stratiotes* biomass in 250 mL glass beaker filled with 100 mL distilled water. The experiments were then incubated at different time intervals of 15, 30 and 60 minutes. After each pre–treatment, the hydrolysates were cooled to room temperature and filtered using a whatman filter paper (0.45 µm). The resulting hydrolysate were analysed for sugar content using a spectrophotometer (Spectroquant R Pharo 300, Merck Germany) at a wavelength of 490 nm, following a phenol-sulphuric acid method (Albalasmeh et al., 2013). The solid biomass retained on the filter paper was washed with distilled water to neutralise pH and air dried at room temperature for further analysis using scanning electron microscopy (SEM). All the experiments were carried out in triplicate and the different pre–treatment time and temperature were selected based on the economic feasibility and previous literature (Harun et al., 2011, Barakat et al., 2014, Barakat et al., 2015, Sun et al., 2016).

3.2.4 Acid pre–treatment

The effect of acid pre–treatment on the hydrolysis of *P. stratiotes* was studied under both boiling (100°C) and steaming (121°C) temperature. Both pre-treatments were carried out by mixing approximately 1g dried powdered *P. stratiotes* in 100 mL sulphuric acid (H_2SO_4) solutions with varying concentrations of 1.5%, 2.5% and 3.5% H_2SO_4. The experiments were incubated at different time intervals of 15, 30 and 60 minutes. The different pre–treatment acid concentrations, reaction time and temperature were decided based on the economic feasibility and previous literature as indicated in section 3.2.3 (Harun et al., 2011, Ganguly et al., 2013, Singh and Bishnoi, 2013, Akanksha et al., 2014). Both acid–boiling experiments using a water bath and acid–steaming using an autoclave were performed as discussed in section 3.2.3 in triplicate

3.2.5 Evaluation of *P. stratiotes* morphology using SEM

A scanning electron microscope (SEM) (ZEISS ultra Plus FEG-SEM) at 5.00 kV was employed to examine the surface details of the plant specimen before and after treatment. The samples were dried and mounted on a stainless steel stub with a double–sided tape, and coated with a thin layer of gold to improve electron conductivity and image quality (Kristiani et al., 2013)

3.3 Results and discussion

3.3.1 Raw aquatic plant composition

The biomass composition of *P. stratiotes* used in this study are presented in table 4. The percentage of contents were 97.2% moisture, 21% ash, 1.14% insoluble lignin, 3.94% soluble lignin, 5.26% hemicellulose, and 37.83% cellulose. The hemicellulose and cellulose composition obtained was different from that of *Eichhornia crassipes*, containing 17.3% for cellulose, and 24.7% for hemicellulose (Chuang et al., 2011) and comparable to the composition of *P. stratiotes* obtained by (Sivasankari and David Ravindran, 2016). These results comply with the lignocellulosic materials that retain carbohydrates in the form of cellulose (35 – 45%) and hemicellulose (25 – 40%) (Qiu et al., 2017). The lignin fraction is associated with the holocellulose substance by intermolecular linkages. Hence the biomass was subjected to pre–treatment to break down the heteropolymeric structure of lignocellulose complex (Kumar et al., 2015) (Table 4).

The moisture content of *P. stratiotes* was comparable to *Eichhornia crassipes* (Bhattacharya et al., 2016) and was higher (97.2% ± 0.1) than other lignocellulosic biomass such as bagasse (8.18 ± 0.35%) (Reddy, 2016) and *Azolla filicuilodes* aquatic plant of 11% (Miranda et al., 2016a). Optimum moisture content is important for microorganisms to ensure prolonged metabolism. However, when the water content is too high it can lead to process failure thus minimal drying of the biomass is required prior to fermentation (Fadairo and Fagbenle, 2014, Sukasem and Prayoonkham, 2017). Studies have also reported improved sugar yield and methane production for dried aquatic plant biomass compared to fresh biomass (Sukasem and Prayoonkham, 2017).

The lignin content of *P. stratiotes* was lower than that of reed canary grass (9.5%), sweet sorghum (17.6%), and corn stalk (21.52%) (Kumar et al., 2015). However, characteristics such as the low lignin content along with the high cellulose could result in obtaining more carbohydrates during substrate degradation via hydrolysis (Argun et al., 2017). The results of acid soluble and insoluble lignin of 3.94% and 1.14% are comparable to those of *Eichhornia crassipes* (Chuang et al., 2011). The C: N ratio of 25.7 was within the range for anaerobic digestion which has been reported to be between 15 – 30:1 (Matheri et al., 2017) (Table 4).

Table 4. *Pistia stratiotes* composition

Component	Percentage (%)
Moisture	97.2 ± 0.1
Ash (w/w, dry weight (DW) basis)	21 ± 0.3
Cellulose (w/w, DW basis)	37.83
Hemicellulose (w/w, DW basis)	5.26
Acid soluble and insoluble lignin (w/w, DW basis)	3.94 and 1.14
COD	9658.2 mg/L ± 0.5
Total Nitrogen	375 mg/L ± 0.02
C: N	25.7

3.3.2 Effect of boiling treatment

The effect of boiling treatment (100°C) on sugar yield was investigated at different reaction times of 15, 30, 60, and 90 min (Table 5). An increase in boiling time from 15 to 90 min resulted in an enhancement in the sugar yield from 27.4 to 40.8 mg/g, respectively (*r*: 0.9938, *p*: 0.0709). The results indicated that boiling enhanced the delignification process compared to the untreated biomass (20.9 ± 0.8) concentration. However, the boiling treatment requires prolonged reaction /incubation time to allow extensive penetration of boiling water into the cell structure leading to breakdown of molecular bonds and hemicellulose and cellulose solubilisation. Reaction time and temperature are reported to be the main factor in hot water hydrolysis (Kim et al., 2015b). Aquatic weed *Laminaria japonica* showed improved sugar yield when the temperature was increased from 150°C to 170°C for 20 min (Jung et al., 2011). A recent study reported a significant increase in glucose of 36% to 61% from hardwood when the boiling pre–treatment temperature was increased to 200°C (Kim et al., 2015a). This observation suggested that the application of 100°C was not sufficient enough to hydrolyse the compact lignocellulosic structure of the *P. stratiotes*. The temperature plus short reaction time may not completely delignify the lignocellulose structure but can dissolve some hemicellulose and increase susceptibility of cellulose as evidenced in this study (Kristiani et al., 2013, Delbecq et al., 2018, Rafieenia et al., 2018a). Higher temperatures above 100°C are considered highly effective for complete solubilisation in hydrolysis thus boiling treatment is limited by requirement of longer retention time (Bundhoo et al., 2015) that can increase the overall cost and efficiency of biohydrogen production.

3.3.3 Effect of steaming treatment (121˚C)

The effect of steam pre–treatment at 121˚C on sugar yield at different reaction times of 15, 30, and 60 min were investigated. An increase in steaming time from 15 to 60 min caused a

decline in sugar yield from 54.0% to 40.9%, respectively (*r*: -0.8729, *p*: 0.3244) (Table 5). Steaming treatment has similar effect as boiling but with added advantage of the pressure effect which improves hydrolysis (Elliston et al., 2015). Previously, steam explosion improved digestibility of spruce wood chips by 90% compared to boiling treatment without pressure (Pielhop et al., 2016). The improvement can be attributed to the weakening effect on the lignocellulose structure by boiling effect followed by size transformation to nanoparticles through the effect of pressure (Yunos et al., 2012). The decline in sugar yield as the reaction time increased can be related to the severity of cellulose breakdown leading to degradation of the released reducing sugars since the *P. stratiotes* biomass has less lignin content and not very recalcitrant. A previous report on reed straw indicated a reduction in cellulose percentage from 55% to 46.5% when the steaming temperature and reaction time was increased demonstrating partial sugar degradation (Hu et al., 2013).

Table 5. Effect of pre–treatment at boiling (100°C) and steaming temperature (121°C)

Reaction time (min)	Sugar yield at (mg/g)	
	boiling (100°C)	steaming(121°C)
15	27.4 ± 0.5	54.0 ± 0.1
30	33.4 ± 1.0	57.0 ± 1
60	41.3 ± 0.7	40.9 ± 3
90	40.8 ± 1.0	–

3.3.4 Effect of sulphuric acid treatment at boiling (100˚C) temperature

The effects of different acid concentrations 1.5%, 2.5%, and 3.5% H_2SO_4 and reaction time (15, 30 and 60 min) on sugar yield was evaluated. It was noted that at 1.5% H_2SO_4, an increase in reaction time from 15 to 60 min enhanced sugar yield from 73.6 to 96.2 mg/g, respectively (*r*: 0.9731, *p*: 0.1480) (Table 7). This result indicated that an increase in hydrolysis up to 60 min improves the degradation of the lignocellulose structure. Previous report on the effect of hydrolysis reaction time on lignocellulosic biomass of *Tacca* and *Tigernut* concluded that sugar quantity greatly depends on reaction time (Ofoefule et al., 2015). However, at 2.5% H_2SO_4, an increase in reaction time from 15 to 60 min caused a decline in sugar yield from 115.2 to 106.9 mg/g, respectively (*r*: -0.1804, *p*: 0.8845). This could be as a result that some of the released sugars gets degraded to other compounds at higher acid concentration resulting in reduced total sugar yield (Jönsson and Martín, 2016). Prolonged acid boiling pre–treatment time was previously reported ineffective and promoted degradation of already released monosaccharides to various products such as furfural and hydroxymethylefurfural (HMF) leading to a reduction in total sugar concentration after the

pre–treatment (Harun et al., 2011). On the other side the released products can be toxic to the growth of microorganisms and inhibit fermentation process (Bundhoo et al., 2015). Similarly, at 3.5% H_2SO_4, an increase in reaction time from 15 to 60 min caused a decline in sugar yield from 112.2 to 95.2 mg/g, respectively (*r*: -0.9365, *p*: 0.2281). It was also noted that an increase in acid concentration from 2.5% to 3.5 % caused an overall decrease in sugar and could be attributed to the degradation of released sugars as explained in section 3.3.4. Similar results were observed in a study by Reddy et al. (2016), which found an increase in H_2SO_4 concentration from 0.64% (v/v) to 1% (v/v) caused a decrease in the sugar yield from 274 to 199.0 mg/g, respectively for bagasse.

3.3.5 Effect of acid treatment plus steaming temperature (121°C)

The effects of different acid concentrations 1.5%, 2.5%, and 3.5% H_2SO_4 on sugar yield at the steaming temperature was investigated. With an increase in acid concentration from 1.5% H_2SO_4 to 2.5% H_2SO_4, an increase in sugar yield from 115 to 139.8 mg/g was observed (Table 6). However, a further increase in acid concentration up to 3.5% H_2SO_4 caused a decline in sugar yield to 130.2 mg/g. This result indicates that the pre–treatment increased removal of hemicelluloses from the solid fraction and enhanced cellulose digestibility, however at or above 3.5% H_2SO_4 steaming, it may promote higher sugar degradation (Argun et al., 2017).

The highest sugar yield of 139.8 mg/g was obtained when *Pistia stratiotes* was exposed to combined treatment of steaming and 2.5% H_2SO_4 at a reaction time = 30 min. Under this condition, the high steaming temperature might have increased the surface area of the substrate that allowed an improved acid penetration into the sample resulting in a complete delignification of the plant biomass (Harun et al., 2011). Reddy et al. (2016) found that contact time was the main factor affecting sugar yield from sugarcane bagasse, whereas Xia

et al. (2013) reported that the rate of degradation of the lignocellulosic structure depended on temperature and concentration of sulphuric acid. This variation in result concludes that the effectiveness of pre–treatment depends not only on the pre–treatment conditions but also on the type of the biomass material.

Table 6. Effect of sulphuric acid treatment at boiling (100°C) and steaming temperature (121°C)

Sulphuric Acid concentration (%)	Reaction time (min)	Sugar yield at (mg/g)	
		boiling (100°C)	steaming(121°C)
1.5	15	73.6 ± 0.8	126.8 ± 0.8
1.5	30	76.1 ± 2.1	135.3 ± 0.6
1.5	60	96.2 ± 1.0	136.1 ± 3.1
2.5	15	115.2 ± 0.2	131.0 ± 1.6
2.5	30	92.6 ± 0.2	139.8 ± 0.8
2.5	60	106.9 ± 0.8	123.6 ± 2.5
3.5	15	112.2 ± 0.2	131.2 ± 0.3
3.5	30	101.2 ± 0.1	115.0 ± 0.3
3.5	60	95.2 ± 2.2	130.4 ± 3.5

3.3.6 Evaluation of *P. stratiotes* morphology using SEM

Scanning electron microscopic analysis was used to view the morphological modifications caused by the different hydrolysis pre-treatment methods. The SEM image of untreated *P. stratiotes* revealed that holocellulose substances (i.e. cellulose and hemicellulose) were coated and closely attached to lignin (figure 4). The SEM image (Figure 4c) of acid treated biomass with 2.5% H_2SO_4 indicated that after acid pre–treatment, the surface was degraded

completely leading to the breakdown of the recalcitrant heteropolymeric fractions of the lignocellulose complex. The change in biomass morphology suggested that the inner materials were ruptured to liberate a large quantity monomeric carbohydrate (figure 4c).

The SEM image of acid treated biomass with 3.5% H_2SO_4 showed a major structural damage which indicate that the treatment completely hydrolysed the plant material. However, the major structural damage indicates a possibility of cellulose and hemicellulose degradation. Extensive structural damage was observed on wheat straw, Hemp, Amor silver grass pre–treated with N_2 steam explosion pre–treatment at 200°C compared to 150°C (Tutt, 2015) and disorganisation on the morphological surface of reed straw caused by steam explosion pre-treatment were also observed under SEM (Hu et al., 2013). The changing morphology of biomass can be related to the effectiveness of the pre–treatment method, the delignification, and the decreasing crystallinity of the cellulose (Kristiani et al., 2013). The SEM results indicate a positive correlation and reliability of the observation between the sugar yield results and the degree of morphological destruction, the highest sugar yield was observed at 2.5% H_2SO_4 for 30 minutes (figure 4).

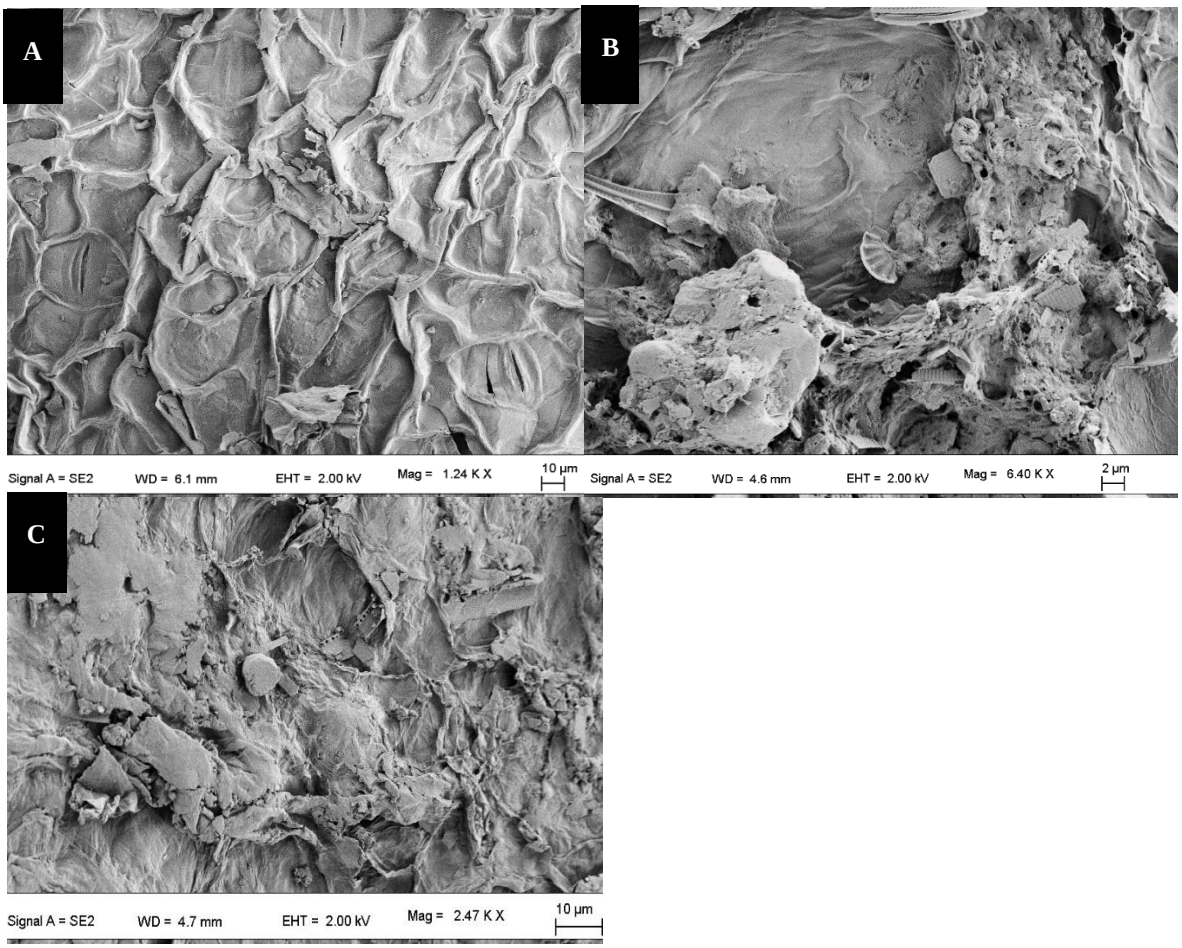


Figure 4. Scanning electron microscope showing micrograph of a) untreated sample b) 3.5% H_2SO_4 treated at steaming temperature for 30 minutes and c) 2.5% H_2SO_4 treated at steaming temperature for 30 minutes.

3.4 Conclusions

- The chemical composition of *Pistia stratiotes* indicated a suitable biomass. The lignin (3.94% and 1.14%) percentage are low with a high cellulose and hemicellulose (37.83%) percentage indicating possible easy solubility of lignin that is sufficient enough to generate fermentable sugars with optimum pre–treatments.
- The hydrolysis pre-treatment process of *P. stratiotes* for 30minutes achieved the maximum respective sugar yield of 139.8 mg/g after steam (at 121°C) pre–treatment with 2.5% H_2SO_4.
- The change in biomass morphology suggested that the inner materials were ruptured to liberate a large quantity of monomeric carbohydrate. Scanning electron microscopic analysis validated the effectiveness of steam pre–treatment of 2.5% H_2SO_4 at 121°C in hydrolysis of *P. stratiotes*. The results showed the correlation between the surface destruction of the biomass and the maximum sugar yield obtained.
- Therefore, the hydrolysate of *P. stratiotes* can further be evaluated for biohydrogen production through dark fermentation process.

CHAPTER IV

OPTIMISATION OF THE REACTOR OPERATIONAL CONDITIONS FOR HYDROGEN PRODUCTION BASED ON CHEMICAL AND MICROBIOLOGICAL ANALYSIS, USING A THERMALLY TREATED ACTIVATED SLUDGE AS AN INOCULUM

4.1 Introduction

Dark fermentation has been extensively explored for biohydrogen production due to its ability to utilize a wide variety of inexpensive substrates, independence of light, and the end–products that can be utilized for photo fermentative H_2 production (Ghimire et al., 2015a, Rai, 2016). However, the challenge in dark fermentation is to optimise for factors affecting H_2 production. Previous studies have suggested that low HYs are ascribed to the type of inoculum used, substrate and operational factors (Kothari et al., 2017, Vi et al., 2017).

For dark fermentative H_2 production to move to the level of commercialization and be applied in industrial scale, the operational factors (type of inoculum, pH, HRT, S/X and temperature) must all together be optimum. The dark fermentation processes and the factors affecting dark fermentation are wholly discussed in chapter II (section 2.3).

Hydrogen productivity depend on the presence of hydrogen producing microorganisms in the fermenter. Hence, this objective focused on the pre–treatment of the anaerobic sludge as a seed inoculum to enrich H_2 producers and eliminate unwanted microorganisms. Biohydrogen fermentation was then estimated at different experimental factors, viz., medium pH, fermentation time, culture temperature, and substrate–to–biomass (S/X) ratio to obtain optimum levels. The reliability of experimental results was examined by employing

COD mass balance and electron–equivalent balance. A regression model was developed to describe the correlation between the studied factors and HYs. The results of H_2 production were then correlated with the microbial community analyses results, including fluorescent in–situ hybridization (FISH), polymerase chain reaction (PCR), and real–time polymerase chain reaction (qPCR).

4.2 Materials and methods

4.2.1 Inoculum preparation

Activated sludge samples were collected in from the anaerobic tank of a domestic wastewater treatment plant in KwaZulu–Natal, South Africa. The samples were collected in 1L screw cap glass bottle and transported to the laboratory under 25°C. Characterization was carried out by using 50 mL of collected sample (activated sludge). pH, total solids (TS), total suspended solids (TSS) and volatile suspended solids (VSS) and mixed liquor suspended solids (MLSS) were analysed according to standard methods (Clesceri et al., 1998) (Appendix one). The 950 mL collected anaerobic sludge in screw cap glass bottle was then thermally treated at 100°C for 45 minutes (to eliminate the undesired microorganisms from the sludge) using an autoclave (Speedy autoclave, HL-341) (Shaterzadeh and Ataei, 2017). After the pre–treatment, it was allowed to cool down at room temperature and was directly inoculated in the reactor.

4.2.2 Substrate preparation and pre–treatment

Water lettuce (*Pistia stratiotes*) was harvested from a river in Durban, KwaZulu–Natal. The plant was washed with tap water to remove dirt and dust, and then the roots and rotten parts were discarded. The stem and leaves were dried at 105°C for 24 h using an oven (Labcon, South Africa). The dried species were blended and sieved to obtain particle size between 0.25 and 0.5 mm (stainless steel, South Africa). The biomass was thereafter subjected to pre–treatment process to increase the release of amount of monomeric sugars. For this, the samples were soaked in sulphuric acid solutions of (v/v) 2.5% for 30 min at a temperature 121°C. After biomass treatment, the hydrolysates were filtered using a whatman filter paper

(Grade 6, circles, diameter 150 mm) and analysed for sugar using a calorimetric Phenol–sulphuric acid protocol (Albalasmeh et al., 2013) (Dubois, 1956).

4.2.3 Experimental setup for H_2 production

Dark fermentation was conducted using batch experiments in serum bottles 120 mL with a working volume of 90 mL. The bottles were sealed with an aluminium cap and a rubber stopper. The bottles were then placed in an incubator (Labotec EcoTherm, South Africa). The anaerobic condition was ensured by purging the sealed bottles with nitrogen gas. The inoculum sludge's pH was 7.2, and the concentrations of Volatile suspended solids (VSS) and total suspended solids (TSS) were 2.4 – 2.6 g/L and 3.2 – 3.5 g/L, respectively. The mixed culture was fed with 60 mL substrate of different COD concentrations (0.6 – 2.9 g/L) depending on S/X required. The mineral medium was composed of (per 1 L) (Reddy et al., 2017): 80 g NH_4HCO_3, 1.24 g KH_2PO_4, 0.1 g $MgSO_4$, 0.0278 g $FeCl_2$, 0.01 g NaCl, 0.01 g $Na_2MoO_4 \cdot 2H_2O$, 0.01 g $CaCl_2 \cdot 2H_2O$, and 0.0015 g $MnSO_4 \cdot 7H_2O$.

The initial standard values were kept at HRT 6, pH 6.5, S/X 1, Temperature 25°C and A one-factor-at-a-time method was employed to investigate the effects of pH: 4.5, 5.5, 6.5, 7.5, and 8.5 (1st experiment), contact time: 4, 6, 8, 10, and 12 h (2nd experiment), temperature: 15, 20, 25, 30, and 35°C (3rd experiment), and S/X: 0.5, 1.0, 1.5, 2.0, and 2.5 g-COD/g-VSS (4th experiment) on fermentative hydrogen production. The optimum condition obtained from each run was used for the next experiment. All experiments were conducted independently in duplicate, and the results were averaged. At the end of each batch experiment, the produced gas was collected using a gas-tight syringe, measured by water displacement method and further analysed composition using gas chromatography (GC-2014, Shimadzu South Africa (Pty) Ltd) equipped with a thermal conductivity detector (Nasr et al., 2015).

4.2.4 Electron equivalent balance

Hydrogen production and metabolic end–products of the fermentation process were converted into electron–equivalent (e^- eq) using Eq. (12). (Amin et al., 2016).

$$e^-_{glucose\ initial} \rightarrow e^-_{glucose\ final} + e^-_{SMP} + e^-_{H2} + e^-_{biomass} \quad (12)$$

where, $e^-_{glucose,initial}$ is the e^- eq of initial glucose Eq. (12), $e^-_{glucose,eff}$ is the e^- eq of remaining glucose Eq. (13), e^-_{SMP} is the e^- eq of soluble microbial products Eqs. (14 – 17), e^-_{H2} is the e^- eq of the cumulative biohydrogen gas Eq. (18), and $e^-_{biomass}$ is the e^- eq of biomass growth Eq. (19).

The half reactions of electron donor, electron acceptors, and cell synthesis are presented as follows (Lee and Rittmann, 2009):

For electron donor:

$$e^-_{glucose} \rightarrow \left(\frac{1}{4}\right) CO_2 + H^+ + e^- \rightarrow \left(\frac{1}{24}\right) C_6H_{12}O_6 + \left(\frac{1}{4}\right) H_2O \quad (13)$$

For electron acceptor

$$e^-_{glucose} \rightarrow \left(\frac{1}{8}\right) CO_2 + \left(\frac{1}{8}\right) HCO_3^- + H^+ + e^- \rightarrow \left(\frac{1}{8}\right) CH_3COO^- + \left(\frac{3}{8}\right) H_2O \quad (14)$$

$$e^-_{butyrate} \rightarrow \left(\frac{1}{5}\right) CO_2 + \left(\frac{1}{20}\right) HCO_{3^-} + H^+ + e^- \rightarrow \left(\frac{1}{20}\right) CH_3CH_2CH_2COO^- + \left(\frac{7}{20}\right) H_2O \quad (15)$$

$$e^-_{propionate} \rightarrow \left(\frac{1}{7}\right) CO_2 + \left(\frac{1}{14}\right) HCO_{3^-} + H^+ + e^- \rightarrow \left(\frac{1}{14}\right) CH_3CH_2COO^- + \left(\frac{5}{14}\right) H_2O \quad (16)$$

$$e^{-}_{lactate} \rightarrow \left(\frac{1}{6}\right) CO_2 + \left(\frac{1}{12}\right) HCO_{3^-}\ H^+ + e^- \rightarrow \left(\frac{1}{12}\right) CH_3CHOHCOO^- + \left(\frac{1}{3}\right) H_2O$$

(17)

$$e^-{}_{H2} \rightarrow H^+ + e^- \rightarrow (1/2)H_2$$

(18)

For cell synthesis

$$e^{-}_{biomass} \rightarrow \left(\frac{1}{5}\right) CO_2 + \left(\frac{1}{20}\right) NH_{4^+} + \left(\frac{1}{20}\right) HCO_{3^-} + H^+ + e^- \rightarrow \left(\frac{1}{20}\right) C_5H_7O_2N + \left(\frac{9}{20}\right) H_2O$$

(19)

The accumulation of biomass was estimated by Eq. (20).

$$\Delta_{biomass} \rightarrow (tCOD - sCOD)_{final} - (tCOD - sCOD)_{intial}$$

(20)

where, $\Delta_{biomass}$ is the biomass accumulation (mg/L), tCOD and sCOD are total and soluble COD, respectively (mg/L), and the chemical formula for biomass is assumed as $C_5H_7O_2N$ (i.e., 1 mol biomass has 160 g COD)

4.2.5 Stoichiometry of H_2 production

The stoichiometric oxidation–reduction equation was established by Eq. (21). (Reddy et al., 2017).

$$R_{overall} \rightarrow f_e \cdot R_a + f_s \cdot R_C - R_d$$

(21)

where, $R_{overall}$ is the balanced reaction, R_a, R_c, and R_d are the half reactions for electron-acceptor reduction, cell synthesis, and electron-donor oxidation, respectively, f_e is the

fraction of electron donor moved to the electron acceptor, and f_s is the portion of donor electrons transferred to cell synthesis, in which $f_e + f_s = 1$.

The half reaction of mixed electron acceptors is given by Eq. (22) (Amin et al., 2016).

$$R_a = \sum_{i=1}^{n} e_{ai} R_{ai} \left(e_{ai} = \frac{e^-_{eq,i}}{\sum_{i=1}^{n} e^-_{eq,i}} \; and \sum_{i=1}^{n} e^-_{a,i} = 1 \right) \tag{22}$$

where, e_{ai} is the fraction of e^- eq for i product of total e^- eq for SMP and H_2, R_{ai} is the half reaction for reduction of i product, and $e_{eq,i}$ is e^- eq of i product. The following conversions were used: 1 mol acetate = 8 e^- eq; 1 mol butyrate = 20 e^- eq; 1 mol propionate = 14 e^- eq; 1 mol lactate = 12 e^- eq;1 mol of biomass as $C_5H_7O_2N$ = 20 e^- eq; and 1 mol H_2 = 2 e^- eq. Procedures are presented in Appendix one.

4.2.6 Analytical methods

The cellulose and hemicellulose components of the dried plant samples were determined by high–performance liquid chromatography (HPLC; Shimadzu, Japan) with a refractive index detector and Aminex HPX-87H column (300 × 7.8 mm) at 40 °C. After each batch experiment, the supernatant was withdrawn and filtered using a whatman filter paper (Grade 6, circles, diameter 150 mm). The solution was analysed for total COD, soluble COD, MLVSS, and pH following standard methods (APHA, 1998). Volatile fatty acids (VFAs) components were analysed by gas chromatography (Agilent 7820A GC, Chemetrix (Pty) Ltd; SA) with a flame ionization detector and Zebron™ ZB-FFAP of 30 m × 0.25 mm × 0.25 μm capillary column. Nitrogen was used as a carrier gas, and the oven temperature was programmed at 100°C for 2 min and then increased to 160°C at a ramp rate of 8°C/min for a hold time of 0.5 min. The gas composition was analysed by gas chromatography (GC-

2014, Shimadzu South Africa. (Pty) Ltd) equipped with a thermal conductivity detector and packed column: GS-GasPro (30 m × 0.32 mm ID). Nitrogen gas was used as the baseline, and the temperatures during operation of the oven, detector, and injection port were 80°C, 250°C, and 120°C, respectively. Different standard calibration curves for methane, CO_2 and H_2 were constructed. The regression equation was solved by generating a MATLAB file using the software.

4.3 Microbial analysis

4.3.1 Fluorescent in–situ hybridization (FISH)

Fluorescent in–situ hybridization (FISH) was employed to identify and visualize the H_2–producing microbial community structure within the thermally treated inoculum sludge as described by Levsky et al. (2003).

Sample fixation

Sample was fixed to preserve the cellular morphology. For this the sample (2 mL) was centrifuged at 10,000 x g for 2 minutes, the obtained pellet was washed with a 1X phosphate buffered solution (PBS). The washed pellet was fixed in a 3:1 (v/v) mixture of 4% paraformaldehyde and 1X PBS solution for Gram negative and 70% ethanol was used instead of 4% paraformaldehyde for Gram positive microorganisms. The mixture was incubated overnight at 4°C. After incubation, the sample were spun down and washed the pellet with a 1X PBS. The washed pellet was then re–suspended in 1:1(v/v) mixture of 1X PBS and absolute ethanol, and kept in -20°C for further analysis (hybridization).

Sample Pre–treatment

The fixed samples were subjected to pre-treatment for floc dispersion and permeabilization, to break down the peptidoglycan of Gram positive bacteria and allow sufficient passage of FISH probes. The cells were washed with 1 X PBS to remove the fixative reagents and centrifuged at 500 x g for 5 minutes. 200 µl protease K at different concentration (0.01, 0.05 and 0.1µg/ml), Tris–HCL (20 mM) and $CaCL_2$ (2 mM) were added in the tube and incubated for 10, 15 and 20 minutes at 37°C. The samples were then washed with 1X PBS to remove protease K and used for hybridization (Amidzadeh et al., 2014)

Hybridization

The microscopic slides were washed in alcohol (1% HCL in 70% ETOH) and placed in a coplin jar containing a diluted poly-L–lysin (0.01%, sigma) for 5 minutes, the slides were then drained and dried for 1 hour at 60°C. 5 µl of pre–treated fixed samples were transferred to the Teflon-coated slides and dried for 10 minutes at 46°C. The specimens were dehydrated in an increasing ethanol series for 3 minutes in 50%, 80% and 100%. 9 µl hybridization buffer solution containing 1.8 M NaCl, 1M Tris–HCl and 10% sodium dodecyl sulfate (SDS) prepared according to formamide percentage (Table 7), was dropped onto each well of the slides. 1ul of oligonucleotide RNA probes (Table 7) was then added onto each well. The slides were placed in hybridization tubes prepared by taking a 50 ml Falcon tube folded with a piece of tissue (saturated with a hybridization buffer) and incubated in a hybridization oven overnight at 46°C. After incubation the slides were then washed and incubated in a hybridization tube containing a pre–warmed wash buffer (1 M Tris/HCl, 10 % SDS, 0.5 M EDTA and NaCL) (Table 7) at 48°C for 10 minutes. The slides were then rinsed with cold water, dried and stained with 8 µl DAPI (4',6-diamidino-2-phenylindole) for 5 minutes. The DAPI stained slides were washed with warm water and dried in the dark. The dried slides

were mounted with Vector shield to prevent photo–bleaching. Microscopic imaging was then performed using epifluorescence microscope (Zeiss AxioLab) equipped with (ZEISS Axiocam MRc color camera).

Table 7. FAM labelled oligonucleotide probes

Short probe name	Target group	Probe sequence	Formamide (%)	Reference
EUB 3881	most Bacteria	5'- GCT GCC TCC CGT AGG AGT -3'	0-50	(Alm et al. 1996)
EUB338 (Bact338)	most Bacteria	5'- GCT GCC TCC CGT AGG AGT -3'	0-50	(Alm et al. 1996)
EUB338 III (SBACT V 338)	Verrucomicrobiales	5'- GCT GCC ACC CGT AGG TGT -3'	0-50	(Alm et al. 1996)
BET42a	Betaproteobacteria	5'- GCC TTC CCA CTT CGT TT -3'	35	(Reddy, 2016)
GAM42a	Gammaproteobacteria	5'- GCC TTC CCA CAT CGT TT -3'	35	(Reddy, 2016)
EBAC1790	*Enterobacteriaceae*	5'- CGT GTT TGC ACA GTG CTG -3'	40	(Alm et al.1996)
CHIS 150	*Clostridium*	5'- TTATGCGGTATTAATCTYCCTTT -3'	0	(Reddy, 2016)

4.3.2 DNA Extraction

The total metagenomic DNA was extracted from different isolates using the phenol–chloroform method modified by Awolusi et al. (2016). Briefly, 2 mL of cell culture was withdrawn from the fermenter, washed with double distilled water followed by 1X PBS, and incubated at 65°C for 2 h. After incubation, freeze–thaw was performed to break the cell walls. The protein was removed by adding phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v) and the DNA was precipitated by adding 0.6X volume of isopropyl alcohol. The DNA was then washed with 70% ethanol and suspended in 1X Tris–EDTA buffer. The concentration and purity of DNA were analysed by the NanoPhotometer (Implen GmBH, NP80, Germany). Agarose gel electrophoresis was used to visualize the quality of extracted genomic DNA.

4.3.3 Polymerase chain reaction (PCR)

The primer sets described (Table 8) were used to amplify the extracted genomic DNA in Veriti® 96-Well Thermal Cycler (Applied Biosystems, South Africa (Pty) Ltd). The amplification conditions were: (a) initial denaturation of 95°C for 10 min, (b) 40 cycles of second denaturation at 95°C for 15 second, (c) annealing at different optimum temperature for 30 second, (d) elongation at 72°C for 30 second, and (f) final extension at 72°C for 4 min. The PCR reaction mixture of 25 µl contained 2 µl template DNA, 12.5 µl Power up PCR master mix, 1.5 µl forward primer, 1.5 µl reverse primer, and 7.5 µl nuclease-free water. The amplified PCR products were visualized by agarose gel electrophoresis (1% solution) stained with ethidium bromide. The 100 bp and 1 kb DNA Ladder were used to measure the size of the amplicons. The gel image was captured by G: BOX gel imaging system (Vacutec, South Africa)

4.3.4 quantitative polymerase chain reaction

The population growth of microbial community was quantified by CFX PCR (C1000 Touch™, CFX96, Bio-Rad. Laboratories (Pty) Ltd, USA) using the primer set listed (Table 9).

Separate standard curves were prepared for different primers using purified 16S rRNA gene fragments (target DNA) obtained from PCR–amplified hydrogenase gene (hydA), *Clostridium* spp. and *Enterobacteriaceae* as described by (Jiang et al., 2011, Lienen et al., 2014 and Awolusi et al., 2016). For purification, GeneJET gel extraction kit was used for purification of excised gel slice containing the DNA fragment of target using a clean razor blade. The DNA was cut as close as possible to minimize gel volume. In each sample the concentration in ng/µl was determined using a nanophotometer (Implen GmBH, NP80, Germany). Eq. (23) was then used to calculate the copy number of the targeted species in each of the purified DNA sample. A series of 10–fold dilution was prepared for standard curves and constructed by plotting the qPCR threshold cycle against the copy number.

$$Number\ of\ copies = \frac{16S\ rRNA\ concentration(ng)\ \times Avogradro's\ number}{16S\ rRNA\ amplicon\ size(bp) \times 660(g16S\ rRNA\ mol/\ bp)} \quad (23)$$

In the equation the Avogadro's standard (bp) is (6.02×10^{23}) and 660 is average molecular weight.

The primer set listed in Table 9 were used to carry out qPCR. The reaction mixture of 10 µl in total, contained 2 µl template DNA, 4 µl SYBR green master mix (Bio–Rad), 0.4 µl forward primer, 0.4 µl reverse primer, and 3.2 µl deionized water. Quantification of 16S rRNA gene copy number concentration within the sample was calculated based on the final

quantification value after completing 40 cycles. Negative control contained no genomic DNA and were subjected to the same conditions. The melting curve analysis and gel electrophoresis were used to confirm specificity of each qPCR run. The amplification condition employed (Table 9)

Table 8. Primers for PCR, real–time PCR and isolation used in this study

Primer name and targeted organism	Forward primer (5'–3')	Reverse primer (5'–3')	Product size (bp)	Reference
Bacillus spp.	GAG TTA GAG AAC GGT ATT TAT GCT GC	CTA CTG CCG CTC CAT GAA TCC	199	Reddy et al. (2017)
Clostridium spp.	AGC GTT GTC CGG ATT TAC TG	TTC GCC ACT GGT ATT CTT CC	182	de Sá et al. (2011)
Enterobactericeae	CAG GTC GTC ACG GTA ACA AG	GTG GTT CAG TTT CAG CAT GTA C	512	Reddy et al. (2017)
Hydrogenase	AAG AAG CTT TAG AAG ATC CTA A	GGA CAA CAT GAG GTA AAC ATT G	259	de Sá et al. (2011)
Bacteria (P338 & P518)	ACT CCT ACG GGA GGC AGC AG	ATT ACC GCG GCT GCT GG	236	(Reddy, 2016)

Table 9. Optimized PCR protocols

PCR steps	quantitative PCR conditions		
	Hydrogenase gene (hydA)	*Clostridium* spp.	*Enterobacteriaceae*
Initial activation	10 minutes at 95°C	3.30 minutes at 95°C	3.30 minutes at 95°C
Denaturation	15 seconds 95°C	30 seconds 95°C	30 seconds 95°C
Annealing	1 minutes 58°C	1 minutes 58°C	1 minutes 62°C
Extension	30 seconds 72°C	30 seconds 72°C	30 seconds 72°C
Number of cycles	40	40	40
Melt curve	55-65°C increment of 0.5°C every 10 seconds	55-65°C increment of 0.5°C every 10 seconds	55-65°C increment of 0.5°C every 10 seconds

4.4 Results and discussion

4.4.1 Effect of pH

The effect of different pH values (4.5, 5.5, 6.5, 7.5, and 8.5) on the hydrogenogenic process was investigated while keeping other operating conditions constant (fermentation time: 8 h, temperature: 25°C, and S/X: 1.0 g-COD/g-VSS). The CH_4 gas was not detected in the collected biogas till 8 h, confirming that heat–treated anaerobic sludge inhibited the methanogenic activity.

The results in Table 10 shows that the HY: 2.14 mol-H_2/mol-glucose at solution pH of 4.5, was lower than that at pH of 5.5 (i.e. HY: 2.46 mol-H_2/mol-glucose). A solution pH < 5.0 can sustain VFA accumulation, which causes system instability and inhibits the culture from an efficient substrate utilization (Singh et al., 2013). This observation was confirmed by the electron–equivalent balance, in which the fraction of electrons involved for the mixed–acid fermentation at pH: 4.5 (e^- eq: 59.9% of e^- eq $_{glucose,\ initial}$) was higher than that at pH: 5.5 (e^- eq: 54.9%). Moreover, a highly acidic medium can reduce microorganism's germination and hinder the fermentation process, leading to a decrease in the conversion of glucose into H_2 gas (Shaterzadeh and Ataei, 2017). This result was in agreement with the COD mass balance (Table 10), which indicated that the percentage of biomass growth at pH of 5.5 was 27.7% of total COD $_{influent}$, and then dropped to 22.5% at pH of 4.5.

A further increase in pH from 5.5 to 8.5 caused a reduction in HY from 2.46 to 1.33 mol-H_2/mol-glucose, respectively (r: -0.9427, p: 0.0573). The alkaline condition tends to neutralize the acidogenic medium and delay the bacterial growth responsible for hydrogen fermentation (Reddy et al., 2017). Similarly, the electron sinks accumulated for the mixed–acid fermentation at pH: 8.5 was lower than that at pH: 5.5, implying the neutralization process of the formed intermediates. Moreover, the decrease in hydrogen productivity at the alkaline medium could be due to the distribution of electrons for the biomass growth (e^- $eq_{biomass}$: 30.9%) rather than for hydrogen formation (e^- eq_{H_2}: 9.8%).

The results indicated that the optimum medium pH was 5.5, achieving HPR of 59.6 mmol-H_2/L/d and HY of 2.75 L-H_2/g-COD $_{removed}$. Under this condition, the highest H_2 electron sink of 18.9% of e^- $eq_{glucose\ initial}$ was obtained. Romão et al. (2014) reported that the hydrogenogenic pathways including acetate and butyrate production could be established at solution pH of 4.5 – 6.5, These researchers used Cheese whey permeate as a substrate and anaerobic sludge consisted of a microbial consortium which was confirmed using isolation technique to be dominated by *Clostridium* spp., *Lactobacillus* spp., and *Enterobacter* spp. In another study, Rezania et al. (2017) found that the pH range of 5.5 – 7.0 provided the optimum condition for H_2 fermentation from lignocellulosic hydrolysates, they used Duckweeds as a substrate and the seed sludge was a mixture of anaerobic microorganisms taken from a lab-scale anaerobic bioreactor system. The overall reaction at pH of 5.5 is given by Eq. (24).

$$C_6H_{12}O_6 + 0.2H_2O + 0.24NH^+{}_4 \rightarrow 0.9CH_3COO^- + 0.27CH_3CH_2CH_2COO^- + 0.08CH_3CH_2COO^- + 0.05CH_3CHOHCOO^- + 1.28CO_2 + 0.24C_5H_7O_2N + 1.54H^+ + \mathbf{2.46H_2} \quad (24)$$

(Mass balance errors for C, H, and O are 3.5%, 0.00% and 5.0%, respectively)

The electron equivalent balances describing the residual glucose, soluble products, H_2 production, and cell synthesis ranged between 94.7% and 95.9%. The acetic and butyric acids were the major soluble microbial products (SMP) accumulated in the fermenter. The production of these acids improves the H_2 fermentation process because a maximum of 4 mol-H_2 per mol-glucose is generated when acetic acid is the main metabolite Eq. (25). In addition, when butyric acid is the main fermentation end products, 2 mol-H_2 per mol-glucose is obtained Eq. (26).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (25)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$$

(26)

The COD mass balance including the theoretical COD values for the remaining glucose, soluble metabolites, and biomass was 92.7 – 94.7%. This difference could be attributed to the presence of other soluble metabolites that were not detected during analyses (de Amorim et al., 2012).

Table 10. Effect of pH on fermentative hydrogen production, including end-product distributions, COD mass balance and Electron-equivalent balance

pH	4.5	5.5	6.5	7.5	8.5
Hydrogen production rate (HPR) and hydrogen yield (HY)					
HPR (mmol-H_2/L/d)	51.0±2.7	59.6±6.4	56.7±2.2	34.0±0.8	30.8±2.7
HY (mg-H_2/g-DW)	3.05±0.04	3.51±0.20	3.30±0.06	2.05±0.06	1.89±0.14
HY (mL-H_2/g-DW)	34.1±0.4	39.4±2.3	37.0±0.7	22.9±0.7	21.2±1.5
HY (L-H_2/g-$COD_{removed}$)	2.67±0.16	2.75±0.07	2.09±0.03	0.97±0.09	0.82±0.05
HY (mol-H_2/mol-glucose)	2.14±0.03	2.46±0.14	2.32±0.04	1.44±0.04	1.33±0.10
Electron-equivalent balance					
$Glucose_{final}$ (%)	2.5±0.2	2.3±0.2	1.4±0.1	2.6±0.1	3.2±0.1
Acetic acid (%)	29.4±0.2	27.9±2.4	27.4±0.6	25.4±0.6	23.4±2.7
Butyric acid (%)	23.2±1.2	20.3±0.7	20.2±0.5	18.7±0.7	17.9±0.6
Propionic acid (%)	4.5±0.1	4.5±0.0	4.7±0.0	5.8±0.2	7.1±0.3
Lactic acid (%)	2.8±0.2	2.2±0.2	2.0±0.1	2.7±0.1	2.8±0.2
Biomass (%)	16.1±1.2	19.8±1.8	22.2±0.8	29.8±1.2	30.9±2.9
H_2 (%)	16.2±0.9	18.9±0.6	18.0±0.7	10.8±0.4	9.8±0.5
Electron balance (%)	94.7±3.7	95.9±5.7	95.9±1.7	95.6±1.7	95.0±7.2
COD mass balance					
Soluble COD_{final} (%)	52.0±1.8	43.6±1.3	41.7±0.7	40.5±1.1	40.3±1.2
Biomass as COD (%)	22.5±0.5	27.7±0.9	31.1±0.5	41.7±0.9	43.4±2.4
H_2 as COD (%)	18.3±0.6	21.4±1.3	20.3±0.5	12.2±0.6	11.0±0.2
COD balance (%)	92.8±2.9	92.7±2.1	93.2±1.7	94.4±1.0	94.7±3.4

Constant factors (Fermentation time 8 h, temperature 25°C), and S/X 1.0 g-COD/g-VSS) and ± standard deviation from the mean value.

4.4.2 Effect of fermentation time

The effect of different fermentation periods (4, 6, 8, 10, and 12 h) on the hydrogenogenic process was investigated while keeping other operating conditions as pH: 5.5, temperature: 25°C, and S/X: 1.0 g-COD/g-VSS.

The results in Table 11 revealed that a decrease in fermentation time from 8 to 4 h caused a decline in HY from 2.29 to 2.13 mol-H_2/mol-glucose, respectively (*r*: 0.9974, *p*: 0.0459). A short reaction time can have a negative impact on H_2 production because of the poor utilization of substrates (Hawkes et al., 2007). These findings agreed with the COD mass balance, in which the soluble COD_{final} at the reaction time of 4 h attained the largest percentage (i.e. 53.5%) amongst other fermentation periods. Thus, the fermentation time < 8 h was not sufficient to convert the carbonaceous matter into H_2. However, as time increased from 8 to 12 h, the HY slightly declined from 2.29 to 1.95 mol-H_2/mol-glucose, respectively (*r*: -0.9368, *p*: 0.2276). The long reaction time allows for a better organic utilization due to the efficient contact of substrate with microorganisms (Kumar et al., 2014). However, the e^- eq of biomass growth increased from 22.7% to 34.4% with an increase in time from 8 to 12 h, respectively (*r*: 0.9912, *p*: 0.0843). This result suggested that the long fermentation time could encourage the accumulation of electron sinks for biomass growth rather than for H_2 formation. According to Bravo et al. (2015), a long fermentation time can facilitate the reproduction of slow–growing microorganisms, e.g., methanogenic archaea that consume the hydrogen gas. Moreover, bioH_2 production can be inhibited by the accumulation of organic acids at long incubation time (Sotelo-Navarro et al., 2017).

These results showed that fermentation time of 8 h was appropriate for the acidogenesis phase, in which the fermentative bacteria generate organic acids, and produce H_2 gas. The HY of 1.86 L-H_2/g-$COD_{removed}$ at 8 h was higher than that reported by Singh et al. (2013), i.e. 0.38 L-H_2/g-COD_{added}, using palm oil mill effluent for 16 h. The optimum time is appropriate for minimizing the

accumulation of electron sinks that can divert electrons from bioH_2 generation (Lee and Rittmann, 2009). De Gioannis et al. (2014) found that improved cumulative H_2 production was accompanied by a decrease in overall duration of the fermentation process. In addition, low retention time can depress the H_2–oxidizers such as homoacetogens and methanogens (Lee and Rittmann, 2009). Similarly, Kumar et al. (2014) found that optimum retention time was 6 h, which achieved HPR of 4.49 L/L/d using sugar composition in a continuously–stirred fermenter. The overall reaction at a batch time of 8 h is given by Eq. (27).

$$C_6H_{12}O_6 + 0.01H_2O + 0.27NH_4^+ = 0.90CH_3COO^- + 0.28CH_3CH_2CH_2COO^- + 0.07CH_3CH_2COO^- + 0.02CH_3CHOHCOO^- + 1.18CO_2 + 0.27C_5H_7O_2N + 1.54H^+ + \mathbf{2.29H_2} \quad (27)$$

(Mass balance errors for C, H, and O are 3.7%, 0.00% and 5.3%, respectively)

The results in Table 11 also indicated that the electron–equivalent balances closed within 90.7% to 99.4%, and the COD mass balance was between 91.9% and 94.6%. Acetate and butyrate were the main acidic metabolites, whereas the percentage of propionic acid was relatively insignificant. This result indicated that the culture could undergo the metabolic pathways that favoured hydrogen production (Singh et al. 2013).

Table 11. Effect of fermentation time on fermentative hydrogen production, including end-product distributions, COD mass balance and Electron-equivalent balance

Fermentation time (h)	4	6	8	10	12
Hydrogen production rate (HPR) and hydrogen yield (HY)					
HPR (mmol-H_2/L/d)	94.4±2.8	69.1±2.6	58.0±5.0	38.7±2.1	30.0±3.5
HY (mg-H_2/g-DW)	3.04±0.18	3.17±0.07	3.27±0.04	2.87±0.04	2.77±0.06
HY (mL-H_2/g-DW)	34.1±2.0	35.5±0.8	36.6±0.4	32.1±0.4	31.1±0.7
HY (L-H_2/g-$COD_{removed}$)	2.44±0.13	2.61±0.09	1.86±0.07	1.30±0.07	1.15±0.03
HY (mol-H_2/mol-glucose)	2.13±0.13	2.22±0.05	2.29±0.03	2.01±0.03	1.95±0.04
Electron-equivalent balance					
$Glucose_{final}$ (%)	4.1±0.2	3.0±0.1	2.4±0.1	2.0±0.0	2.1±0.1
Acetic acid (%)	27.1±1.9	28.5±1.0	29.0±0.5	25.1±0.4	20.5±0.4
Butyric acid (%)	19.5±1.5	21.3±1.0	22.4±0.9	18.3±0.6	16.3±0.3
Propionic acid (%)	5.9±0.1	5.1±0.3	3.9±0.1	4.9±0.2	5.3±0.2
Lactic acid (%)	3.6±0.2	1.1±0.0	0.7±0.0	0.4±0.0	1.3±0.0
Biomass (%)	15.4±0.3	18.5±0.5	22.7±0.9	29.9±1.4	34.4±1.2
H_2 (%)	15.0±0.2	16.4±0.5	18.4±0.6	15.3±0.2	14.3±0.6
Electron balance (%)	90.7±4.0	93.8±1.0	99.4±3.0	96.1±1.5	94.2±1.9
COD mass balance					
Soluble COD_{final} (%)	53.5±1.3	47.8±0.6	41.7±0.6	35.1±0.3	30.3±0.9
Biomass as COD (%)	21.5±0.3	25.9±0.8	31.8±1.2	42.0±2.9	48.2±3.6
H_2 as COD (%)	16.9±0.7	18.6±0.7	20.8±1.1	17.3±0.4	16.1±1.2
COD balance (%)	91.9±2.2	92.3±1.8	94.3±2.1	94.4±3.0	94.6±5.6

Constant factors (pH: 5.5, temperature: 25°C), and S/X: 1.0 g-COD/g-VSS) and ± standard deviation from the mean value.

4.4.3 Effect of temperature

The effect of different temperature values (15, 20, 25, 30, 35°C) on the hydrogenogenic process was investigated while keeping other operating conditions as pH: 5.5, fermentation time: 8 h, and S/X: 1.0 g-COD/g-MLVSS (Table 12). Recent studies on fermentative H_2 production from agricultural waste have been conducted under thermophilic temperature (42 – 75°C) for a better hydrolysis of cellulosic compounds (Chuang et al., 2011). However, this study used a pre–hydrolysis step to increase the availability of fermentable sugar.

The findings in Table 12 shows that an increase in temperature from 15 to 25°C resulted in an enhancement of HY from 2.05 to 2.29 mol-H_2/mol-glucose, respectively (*r*: 0.9820, *p*: 0.1210). The bioH_2 production was improved with an increase in temperature, which could be due to the enhancement of the metabolic activity of bacteria (Sotelo-Navarro et al., 2017).

A further increase in temperature from 25 to 35°C caused a slight improvement of HY from 2.29 to 2.36, respectively (*r*: 0.9245, *p*: 0.2490). In addition, a large sink of e^- $eq_{biomass}$ at 35°C was observed for biomass growth, contributing to 25.3% of e^- $eq_{glucose,\ initial}$. The high conversion of substrate for biomass growth at 35°C could be because the bioactivity increases with rising the culture temperature (Qiu et al., 2017). However, additional energy is required for keeping the fermenter at 35°C, which limits the application of the system at a large scale. Hence, this study suggested a room temperature (around 25°C) for bioH_2 fermentation. At a temperature of 25°C, H_2 reached an electron accumulation of 17.3% of $e^-_{glucose,\ initial}$. In addition, a large fraction of electrons transferred to acetic acid (24.8%) and butyric acid (19.8%), indicating that the fermentation pathway was acetate + butyrate type. The overall reaction at temperature of 25°C is given by Eq. (28).

$$C_6H_{12}O_6 + 0.28NH_4 \rightarrow 0.82CH_3COO^- + 0.26CH_3CH_2CH_2COO^- + 0.09CH_3CH_2C00^- + 0.06CH_3CHOHCOO^- + 1.190CO_2 + 0.28C_5H_7O_2N + 1.51H^+ + \mathbf{2.29}\boldsymbol{H}_2 \quad (28)$$

(Mass balance errors for C, H, and O are 3.5%, 0.0% and 5.0%, respectively)

In a similar study, Chuang et al. (2011) found that the cumulative H_2 production was significantly higher when the temperature was over 45°C. Liu et al. (2008) reported 60°C as a favourable temperature for the conversion of microcrystalline cellulose (10 g/L) and corn stalk powder (0.5%) into H_2 using a co–culture of *Clostridium thermocellum* JN4 and *Thermoanaerobacterium thermosaccharolyticum* GD17. In addition, Qiu, et al. (2017) found that the maximum H_2 production of 328.4 mL and HY of 1.31 mol-H_2/mol-$xylose_{consumed}$ were observed at 55°C. The variation in results could be due to the different bacterial species responsible for H_2 production, and the dependence of their growth on certain temperature conditions.

The electron equivalent and COD mass balances of 91.0 – 98.5% and 93.6 – 98.6%, respectively revealed the reliability of the fermentation data. The COD-mass balance was lower than 100%, which could be due to the involvement of a substrate fraction for storage and cell maintenance (Ghimire et al., 2015). In addition, results indicated that H_2 production occurred via the metabolic pathway to produce acetic and butyric acids.

Table 12. Effect of temperature on fermentative hydrogen production, including end-product distributions, COD mass balance and Electron-equivalent balance

Temperature (°C)	15	20	25	30	35
Hydrogen production rate (HPR) and hydrogen yield (HY)					
HPR (mmol-H_2/L/d)	60.5±5.6	64.3±3.1	72.9±9.0	76.4±7.7	81.2±7.8
HY (mg-H_2/g-DW)	2.93±0.05	3.15±0.06	3.27±0.09	3.28±0.04	3.37±0.22
HY (mL-H_2/g-DW)	32.8±0.6	35.3±0.6	36.6±1.1	36.8±0.4	37.7±2.4
HY (L-H_2/g-$COD_{removed}$)	0.94±0.02	0.95±0.01	1.11±0.03	1.10±0.03	1.17±0.02
HY (mol-H_2/mol-glucose)	2.05±0.04	2.21±0.04	2.29±0.07	2.30±0.03	2.36±0.15
Electron-equivalent balance					
$Glucose_{final}$ (%)	5.2±0.5	4.3±0.1	3.9±0.1	0.0±0.0	0.0±0.0
Acetic acid (%)	23.6±1.9	24.5±1.9	24.8±0.8	25.8±1.7	27.8±1.4
Butyric acid (%)	18.5±1.2	19.1±0.6	19.8±0.6	21.0±1.0	21.8±1.5
Propionic acid (%)	5.9±0.6	4.3±0.2	4.7±0.1	4.0±0.2	3.7±0.1
Lactic acid (%)	3.8±0.4	1.0±0.0	2.7±0.1	2.5±0.1	0.7±0.0
Biomass (%)	21.2±1.0	22.6±1.4	23.5±1.6	24.4±0.1	25.3±1.6
H_2 (%)	14.4±1.0	15.3±0.9	17.3±1.5	18.1±1.2	19.3±0.2
Electron balance (%)	92.7±6.3	91.0±5.0	96.8±4.2	95.8±4.0	98.5±4.5
COD mass balance					
Soluble COD_{final} (%)	52.6±2.2	47.7±0.7	43.0±3.9	38.9±2.5	38.9±0.9
Biomass as COD (%)	29.7±0.7	31.7±0.8	32.9±0.3	34.2±1.8	35.4±1.3
H_2 as COD (%)	16.3±0.8	17.3±0.4	19.6±0.3	20.5±0.8	21.8±1.0
COD balance (%)	98.6±3.7	96.7±1.4	95.5±3.9	93.6±3.9	96.1±2.1

Constant factors (pH: 5.5, fermentation time: 8 h, and S/X: 1.0 g-COD/g-MLVSS) and ± standard deviation from the mean value.

4.4.4 Effect of S/X

The effect of different S/X ratios (0.5, 1.0, 1.5, 2.0, and 2.5 g-COD/g-VSS) on the hydrogenogenic process was studied while keeping other operating conditions as pH: 5.5, fermentation time: 8 h, and temperature: 25°C. At S/X of 0.5 g-COD/g-MLVSS, the HPR recorded 41.8 mmol-H_2/L/d, which was lower than that at S/X of 1.5 g-COD/g-MLVSS (i.e. 74.4 mmol-H_2/L/d) (Table 13). At a low S/X ratio, microorganisms mainly utilize the carbon source for cellular respiration and bacterial growth, rather than for bioH_2 production (de Amorim et al., 2012). This result consisted with the data obtained in Table 13, which showed that the electron equivalent for biomass synthesis was high at S/X of 0.5 g-COD/g-VSS with a value of 25.3% of e^- $eq_{glucose,\ initial}$. The sugar content at the optimum S/X ratio can provide an adequate energy source for microorganisms to convert substrate into H_2. The optimum S/X condition is used to maximize e^- eq that transfers to H_2 instead of conserving energy for bacterial metabolism. The overall reaction at S/X of 1.0 is given by Eq. (29).

$$C_6H_{12}O_6 + 0.30NH_4^+ = 0.88CH_3COO^- + 0.27CH_3CH_2CH_2COO^- + 0.05CH_3CH_2COO^- + 0.01CH_3CHOHCOO^- + 1.18CO_2 + 0.30C_5H_7O_2N + 0.04H_2O + 1.52H^+ + \mathbf{2.32H_2} \quad (29)$$

(Mass balance errors for C, H, and O are 3.7%, 0.0% and 5.2%, respectively)

An increase in S/X from 0.5 to 2.5 g-COD/g-MLVSS caused a significant decline in HY from 2.60 to 0.85, respectively (*r*: -0.9860, *p*: 0.0020). A high organic load can become inhibitory to the microorganisms because of pH drop, acidic toxicity, or an increase in the H_2 partial pressure (Ginkel et al., 2001). Bravo et al. (2015) reported that at high substrate concentration, the homoacetogenic bacteria could utilize H_2 and carbon to form acetic acid. Similarly, the e^- eq migrated for acetate production exhibited the highest percentage (28.4% of e^- $eq_{glucose,\ initial}$) at S/X of 2.5 g-COD/g-MLVSS. A previous study by Chuang et al. (2011) found that high S/X could result in an accumulation of VFA, causing inhibitory or toxic effects to the fermentative bacteria. These findings

were in agreement with the COD mass balance, which demonstrated that the highest soluble COD_{final} of 47.6% was obtained at the maximum S/X ratio 2.5 g-COD/g-MLVSS.

The COD mass balance at each S/X ratio exhibited adequate recovery with a maximum error of 8.0% (Table 13), suggesting that the measurements of the degraded substrate and metabolic products were acceptable. Similar findings were obtained for the electron-equivalent balance.

Table 13. Effect of S/X on fermentative hydrogen production, including end-product distributions, COD mass balance and Electron-equivalent balance

S/X	0.5	1	1.5	2	2.5
Hydrogen production rate (HPR) and hydrogen yield (HY)					
HPR (mmol-H_2/L/d)	41.8±1.9	73.6±5.5	74.4±1.8	71.7±4.1	66.6±4.4
HY (mg-H_2/g-DW)	3.70±0.07	3.30±0.16	2.20±0.07	1.63±0.02	1.21±0.02
HY (mL-H_2/g-DW)	41.5±0.8	37.0±1.8	24.7±0.7	18.3±0.3	13.5±0.2
HY (L-H_2/g-$COD_{removed}$)	0.84±0.01	0.91±0.01	0.72±0.00	0.61±0.01	0.57±0.01
HY (mol-H_2/mol-glucose)	2.60±0.05	2.32±0.11	1.55±0.05	1.14±0.02	0.85±0.01
Electron-equivalent balance					
$Glucose_{final}$ (%)	0.7±0.0	2.1±0.1	2.4±0.1	3.0±0.1	5.0±0.2
Acetic acid (%)	25.9±0.3	26.6±1.8	24.9±0.5	27.3±0.3	28.4±0.9
Butyric acid (%)	19.2±0.9	20.7±1.1	23.2±0.6	22.8±0.4	22.5±1.1
Propionic acid (%)	2.2±0.1	2.5±0.2	4.9±0.2	5.7±0.3	4.8±0.2
Lactic acid (%)	1.4±0.0	0.6±0.0	2.7±0.0	0.3±0.0	2.3±0.1
Biomass (%)	25.3±1.3	25.0±1.1	26.2±1.6	27.5±1.0	28.3±1.0
H_2 (%)	19.9±1.3	17.5±0.8	11.8±0.4	8.5±0.1	6.3±0.1
Electron balance (%)	94.5±3.6	95.0±4.8	96.1±1.3	95.2±1.4	97.6±2.1
COD mass balance					
Soluble COD_{final} (%)	35.4±0.7	39.5±3.2	43.1±1.6	44.7±1.7	47.6±0.8
Biomass as COD (%)	35.4±0.4	35.1±1.2	36.7±1.0	38.6±2.0	39.7±2.1
H_2 as COD (%)	22.5±0.6	19.8±1.3	13.3±0.7	9.6±0.3	7.2±0.3
COD balance (%)	93.3±1.4	94.3±5.5	93.2±1.7	93.0±4.0	94.5±2.9

Constant factors (pH: 5.5, fermentation time: 8 h, and temperature: 25°C) and ± standard deviation from the mean value.

4.4.5 Regression model

A pure–quadratic model Eq. 30. was developed to demonstrate the effects of experimental factors on the hydrogen yield.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 \quad (30)$$

where, Y is the model response (hydrogen yield), X_i are the experimental factors (X_1: pH, X_2: time, X_3: temperature, and X_4: S/X), β are the regression model coefficients (β_0, β_i, and β_{ii} are intercept, linear effect, and quadratic effect), and k is the number of factors.

The findings in Table 14 revealed that the coefficient of determination between the measured and estimated results was R^2: 0.928. The high R^2-value revealed a reasonable agreement of the regression equation with the experimental findings. This observation indicated that the percentage of the response variable variation described by the model was 92.8%; however, the quadratic model could not explain 7.2% of differences in the data. In addition, the adjusted-R^2 of 0.875 was comparable to the R^2-value, suggesting that the input factors were important and no data overfitting occurred (Ateia et al., 2016). As listed in Table 14, the linear and quadratic effects of each independent variable on the HY were determined by *t*-test. The solution pH provided a positive linear effect ($p < 0.1$), as well as, a negative quadratic effect ($p < 0.1$) on the model output. This result suggested that the HY could be enhanced by an increase in pH until a peak value, and then started to decline. In addition, the variable "S/X" exhibited a negative linear influence on the response, suggesting that the HY could be increased with a decrease in S/X values. This result indicated that the plot of HY versus each of pH and S/X resulted in quadratic linear concave up and linear down curves, respectively. Moreover, flat curves were obtained for the plot of HY against each of fermentation time and culture temperature. Based on the aforementioned results, the highest HY of 2.96 mol-H_2/mol-glucose could be obtained at pH: 5.3, time: 6.4 h, temperature: 35°C, and S/X: 0.5.

These results suggested that the proposed model could be used to predict the HY at certain independent variables.

Table 14. t-statistics and p-values for coefficients of the pure-quadratic model used for the prediction of HY via dark fermentation of *Pistia stratiotes*

	Beta	SE	t-stat	p-value	Effect
Constant	-0.378	2.220	-0.170	0.868	Non-significant
pH	1.077	0.558	1.929	0.080	Significant
Time	0.137	0.141	0.978	0.349	Non-significant
Temp.	0.018	0.069	0.257	0.802	Non-significant
S/X	-1.077	0.531	-2.028	0.068	Significant
$(pH)^2$	-0.102	0.042	-2.416	0.034	Significant
$(Time)^2$	-0.011	0.009	-1.262	0.233	Non-significant
$(Temp.)^2$	0.000	0.001	-0.051	0.960	Non-significant
$(S/X)^2$	0.032	0.168	0.188	0.854	Non-significant

4.5 Microbial community analysis

4.5.1 Microbial analysis of thermal treated anaerobic activated sludge (using FISH)

The results from fluorescent in situ hybridization analysis are displayed (Figure 5). The microbial community structure of the thermal treated anaerobic sludge indicated the presence of bacterial domain EUB338 mix being reduced in population after the pre–treatment compared to the untreated. This could be attributed to H_2 consumers being eliminated, since they are sensitive to harsh condition while most of the H_2 producing bacteria are spore formers and characterized by tolerance to thermal temperature. Bacteria (such as facultative anaerobes, acidothiobacillus and fermentative bacteria) belonging to the class of *Gammaproteobacteria* and *Betaproteobacteria*, *Enterobacteriaceae* family as well as *Clostridium* genus are characterized by the capability to ferment carbohydrate to H_2, CO_2 and organic acids. These findings revealed the existence of known H_2 producing microorganisms in the seed inoculum (Shaterzadeh and Ataei, 2017).

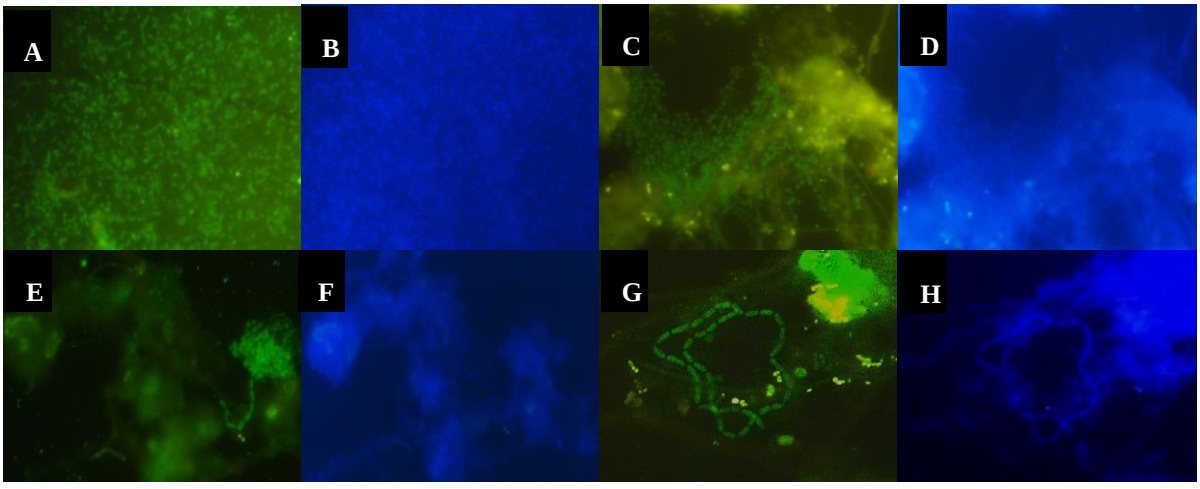


Figure 5. Fluorescence in situ hybridization (FISH) images showing hybridized cells by specific probes and DAPI stained cells: (a) and (b) bacterial domain using EUB338, EUB338II, and EUB338III, (c) and (d) *Enterobacteriaceae* using EBAC1790, (e) and (f) *Gammaproteobacteria* and *Betaproteobacteria* using GAM42a BET42a probes, and (g) and (h) *Clostridium histolyticum* using Chis150.

4.5.2 Polymerase chain reaction (PCR)

PCR was performed for the detection of the hydrogen-producing bacterial species and hydrogenase gene in the reactors using specific primers (Table 8). Figure 6 (a-d) shows the existence of *Bacillus*, *Enterobacteriaceae*, and *Clostridium* as revealed by PCR. The presence of these specific species for H_2 production is directly linked to the activity of hydrogenase gene (Chang et al., 2008). In addition, these species have been reported as the most commonly found facultative anaerobes in H_2 fermentation systems (Mishra et al., 2017). Studies have previously demonstrated a continual presence *Clostridium* and *Enterobacter* species as the hydrogen producing microbial community in dark fermentation reactors (Hung et al., 2008, Laothanachareon et al., 2014, Cisneros-Pérez et al., 2017, Pugazhendhi et al., 2017b). Other studies isolated *Bacillus* from different mixed anaerobic culture as one of the H_2 producers and fermenters (Chaganti et al., 2012, Sutthipattanasomboon and Wongthanate, 2017).

In this study, species such as *Clostridium*, and *Enterobacter* as well as hydrogenase gene (hydA) were detected under all operational conditions, however the *Bacillus* species were not detected when the reactor conditions were adjusted to a pH of 4.5, and which can also be correlated to faint bands observed for the hydA under the same pH conditions indicating low activity of the hydA. *Bacillus* was previously detected in H_2 producing mixed culture under mesophilic conditions (35°C) at 6.5 pH (Mishra et al., 2017b).

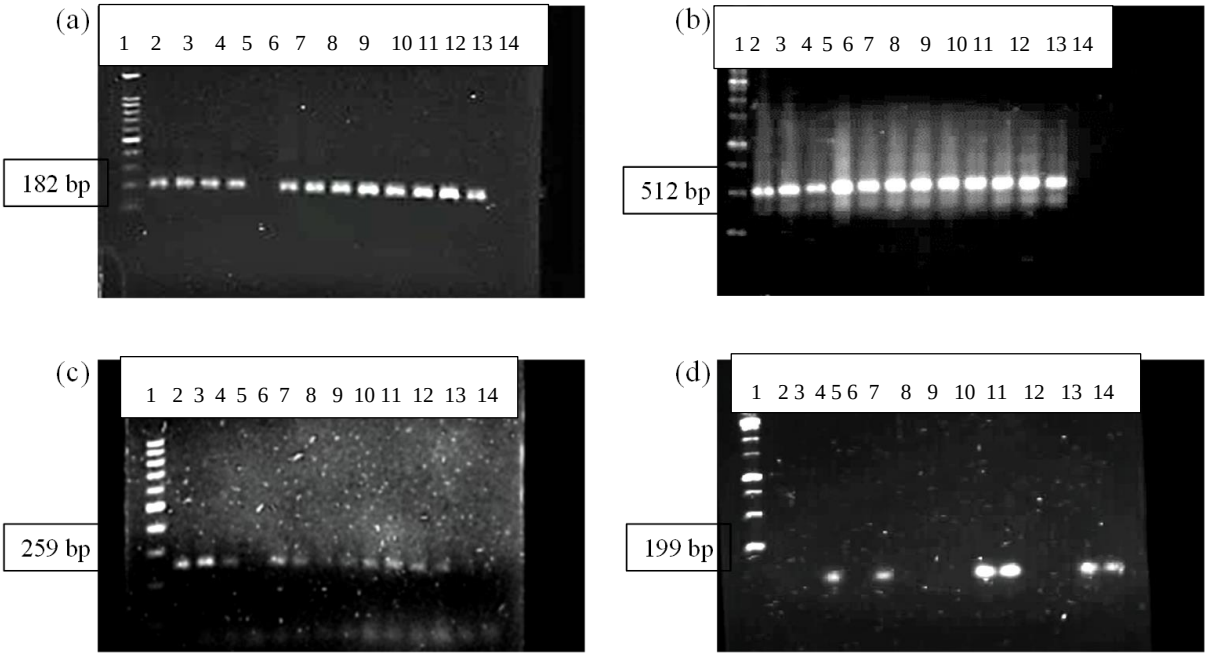


Figure 6. PCR amplification product showing–

(a) (*Clostridium* spp.)– Lane 1 contains the molecular weight ladder. Lane 2-4 contains factor HRT. Lane 5,7,8 contains S/X ratio. Lane 9-11 contains pH. Lane 12-14 contains temperature.
(b) (*Enterobactericeae* (512 bp)–Lane 1 contains the molecular weight ladder. Lane 2-4 contains HRT. Lane 5,7,8 contains S/X ratio. Lane 9-11 contains pH. Lane 12-14 contains temperature.
(c) (Hydrogenase (259 bp) –Lane 1 contains the molecular weight ladder. Lane 2-4 contains HRT. Lane 5,7,8 contains S/X ratio. Lane 9-11 contains pH. Lane 12-14 contains temperature.
(d) (Bacillus spp. (199 bp)– Lane 1 contains the molecular weight ladder. Lane 2-4 contains HRT. Lane 5,7,8 contains S/X ratio. Lane 9-11 contains pH. Lane 12-14 contains temperature

4.5.3 Quantitative Polymerase chain reaction (qPCR)

The abundance of H_2 producing bacteria was analysed using qPCR under different pH regimes of 4.5, 5.5, and 8.5. The hydrogenase gene activity was presented based on a copy number/nanogram of total genomic DNA. As shown in Figure 7(a), the hydA expression provided the highest at pH of 5.5 with a cell number of 2.53×10^4 copies/ng-DNA compared to pH of 4.5 (6.95×10^3 copies/ng-DNA) and 8.5 (7.77×10^3 copies/ng-DNA). Hydrogenase is considered the main enzyme responsible for H_2 production via dark fermentation (Rezania et al., 2017). Similar trends were observed for *Enterobacter* and *Clostridium* spp. using 16SrRNA based primers demonstrating high copy numbers

at pH: 5.5. These species have been recognized as the key H_2 producers because they contain the hydrogenase enzyme responsible for H_2 production (Reddy et al., 2017). This result agreed with the H_2 productivity (Table 10), which indicated the highest HY of 2.46 mol-H_2/mol-glucose at pH of 5.5. Similar results were reported by Won et al. (2013), which found an increased expression of the hydrogenase gene at pH between 5.5 and 6.0.

The variation in fermentation time (4, 8, and 12 h) caused high differences in the activities of hydA and species-specific gene (*Clostridium* spp. and *Enterobacter*). Figure 7(b) shows higher expression of the hydrogenase gene at a reaction period of 8 h (1.90×10^5 copies/ng-DNA) compared to those at 4 and 12 h. Similar, findings were recorded for the potential hydrogenase containing microbes, i.e. *Clostridium* spp. and *Enterobacter*. This result was consistent with the data in Table 11, which depicted that the highest HY was found at a period of 8 h.

Figure 7(c) displays the effect of culture temperature on the hydrogen–producing bacteria. It was observed that an increase in temperature caused an improvement in the functional hydA, recording approximately 2.45×10^5 copies/ng-DNA at 30 – 35°C. In addition, *Clostridium* spp. presented higher copy numbers than *Enterobacter* for all temperature values. The comparison of H_2 producing bacteria at various S/X ratios revealed the dominance of hydA at S/X of 1.0 (Figure 7d). Moreover, the levels of expression of hydA decreased from 1.60×10^4 to 1.23×10^4 copies/ng-DNA with increasing the S/X ratio from 1.0 to 2.5 g-COD/g-MLVSS, respectively. A similar trend was noticed for the species containing a highly active hydrogenase activity (i.e. *Clostridium* spp. and *Enterobacter*). The obtained results agreed with the HYs recorded in Table 14.

Table 15. Parameters of QPCR standard curves for primer sets used in this study

Parameters	Primer		
	Hydrogenase gene(hydA)	*Clostridium spp.*	*Enteobacteriaceae*
Leaners-range (copies/ng DNA)	4.65 x (10^{02}+ 10^{09})	6.03 x (10^{01}+ 10^{08})	7.78 x (10^{01}+ 10^{08})
R^2 slope	0.993	0.981	0.995
Slope (standard deviation)	-3.184	-3.569	-3.372
Intercept	41.654	40.135	37.213

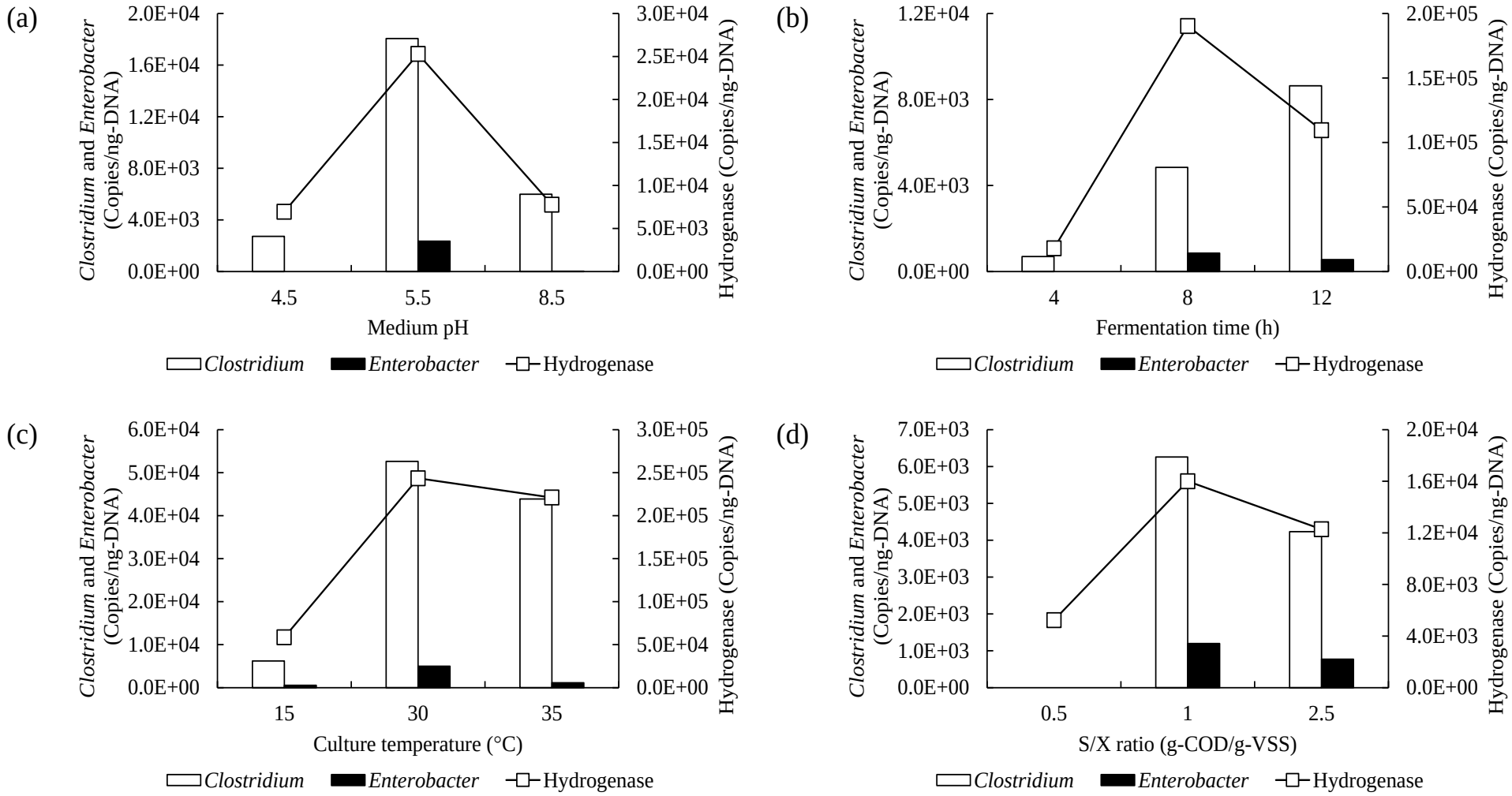


Figure 7. qPCR results for microbial community at different values of (a) medium pH, (b) fermentation time, (c) culture temperature, and (d) S/X ratios.

4.6 Conclusions

The outcome of this objective emphasised the significant of the effect of operational factors that were modelled and optimised (pH, HRT, S/X and temperature) on the enhancement of H_2 production from pre–treated *P. stratiotes*. Based on the results, the following conclusion can be drawn –

- Thermal pre–treatment (100°C, 45 min) of anaerobic sludge was effective in eliminating the H_2 consuming microorganisms in the seed inoculum, whilst promoting the enrichment of spore–forming H_2 producing bacteria, which was confirmed by using FISH with specific probes.
- One–Factor–at–a–time investigation attained optimal operational conditions of– pH 5.5, HRT 8h, temperature 25°C and S/X ratio 1, with a highest HY of 2.46 mol-H_2/mol-glucose (3.51 mg-H_2/g-DW)
- The COD mass balance and electron-equivalent balance indicated the successful transfer of organic carbon to the production of H_2 gas and metabolic intermediates thus the reliability of the aforementioned results
- A quadratic model was developed to predict the HY using the experimental factors as inputs (R^2: 0.928). The solution pH provided positive-linear and negative-quadratic effects on the HY, whereas the plot of HY against S/X showed a negative–linear curve.
- The qPCR results indicated a positive correlation between the abundance of H_2 producing bacteria and H_2 production potential.

CHAPTER V
ISOLATION AND IDENTIFICATION OF THE DOMINANT HYDROGEN PRODUCING BACTERIA FROM THE ENRICHED REACTOR

5.1 Introduction

Selection of efficient microbial candidate is crucial for higher H_2 yield during dark fermentation. Different types of inoculum viz., pure cultures, co–cultures as well as mixed cultures are currently employed for H_2 production (Pachapur et al., 2015, Cabrol et al., 2017b). Mixed cultures from sewage sludge, dairy waste effluent, soil, compost and slaughter house effluent have been used as a potential inoculum source for industrial scale hydrogen production due to their ability to utilise a wide range of organic substrates (Thanwised et al., 2012, Ghimire et al., 2015a, Ghimire et al., 2016, Wang et al., 2017). Presence of huge microbial diversity in mixed cultures makes them a better candidate during large scale hydrogen production (Laxman Pachapur et al., 2015, Wang et al., 2017). Moreover, the presence of a single pure isolate in the system can be advantageous to study the metabolic shifts in the system (Argun et al., 2017). Co–culture system, consist of more than one isolates with different metabolic efficiency combined together to enhance the hydrolysis rate of substrate and to improve the cumulative H_2 production. The advantages and challenges of the above cultures are discussed in chapter II (section 2.3.2.4).

This chapter therefore focused on isolation and identification of dominant H_2 producing bacteria from an enriched H_2 producing reactor using thermally treated mixed culture and aquatic weed *P. stratiotes* as a substrate to develop a hydrogen producing microbial cocktail. H_2 production potential of the isolates were further conducted using batch culture studies in comparison to the thermally treated inoculum sludge.

5.2 Materials and methods

5.2.1 Fluorescent in–situ hybridization (FISH)

Fluorescent in–situ hybridization (FISH) technique was employed to identify and confirm the presence of known H_2 producing bacterial community within the thermally treated inoculum sludge before and after inoculation to the reactor using species specific probes as described in chapter four section 4.3.

5.2.2 Isolation of hydrogen producing strain

One ml sample (log phase) from batch experiments (of enriched mixed anaerobic sludge and *P. stratiotes*) was serially diluted (10^{-1} to 10^{-9}) and plated on Nutrient Agar medium (appendix) and incubated at 30°C overnight in an obligate anaerobic environment, using an anaerobic jar system. After 24–hour incubation, the single colonies were selected and streaked on to fresh Nutrient agar plates. Thereafter, the colonies were selected according to their difference in shape, size and color (Table 16) and sub cultured till the pure (axenic) cultures were established. The pure cultures were then transferred to a liquid nutrient broth and incubated overnight at 30°C and stored at -20°C in 50% glycerol stock for further analysis.

5.2.3 Morphological identification

Morphological analysis was performed with light microscope (Nikon Eclipse 80i) equipped with digital camera (ZEISS Axiocam 105 color) and the Gram staining (Appendix) was performed (Wanger et al., 2017). Gram staining was performed to differentiate bacterial species into gram–positive and gram–negative group as well as their morphology.

5.2.4 16S rDNA analysis

The genomic DNA of the selected isolates was further extracted from the pure colonies employing the phenol–chloroform extraction method as described in chapter 4 (section 4.3.4) (Awolusi et al., 2016). For this few colonies were selected and grown overnight in nutrient broth (Appendix) until it reached OD 600, thereafter, 2 mL of sample was centrifuged down and washed with 1X PBS. After extraction the genomic DNA was further amplified using universal bacteria primer pair set (27f/1492r) and the functional (hydrogenase gene) primer using the thermal cycler (Veriti, Applied Biosystems) (Table 8, chapter 4). The positive amplicons were then identified using 16s rRNA gene sequencing and analysis at a commercial laboratory (Inqaba laboratories, South Africa). The FinchTV software was used for the initial editing and analysis of the sequences and thereafter identified using the BLAST search at National center for biotechnology Information (NCBI) database. A phylogenetic tree was then constructed using MEGA7 software using the selected closely related sequences from NCBI (Hu and Zhu, 2017) using Neighbor joining method (Saitou et al., 1987).

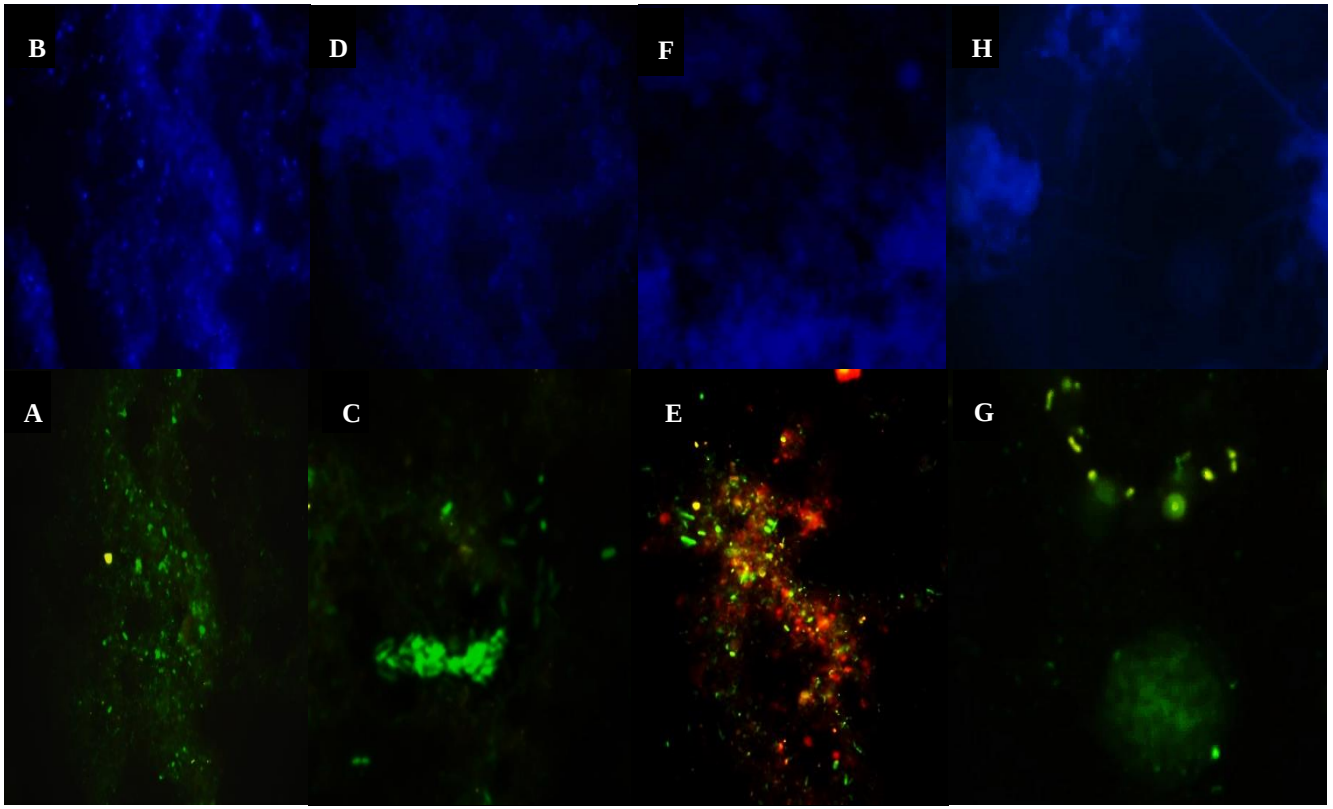
5.2.5 Hydrogen production potential of the isolated strains using *P. stratiotes* as a substrate

To examine H_2 production ability of isolated strains using *P. stratiotes* as a substrate, the anaerobic batch experiments were performed under similar conditions as the optimum mixed culture experiment (chapter 4). For this, 10% (OD600) of active inoculum of the three isolated strains (a) 3_HG-L1F, b) 4_HG-L1F and d) 7_HG-L1F were inoculated in a 120 ml serum bottle containing 90 mL working volume (The serum bottles were 1st flushed with nitrogen gas to create anaerobic environment and sterilized using an autoclave at 121°C for 20 minutes). The experiments were carried under optimized conditions (temperature 25°C, pH 5.5, S/X ratio 1, and reaction time 8 h). A control experiment was also performed in parallel using pretreated anaerobic activated sludge. All the experiments were conducted under optimized conditions as described in chapter 4. Section 4.2.3. Chemical and H_2 gas analysis after the experiment, were performed as described in chapter 4. section 4.2.6.

5.3 Results and Discussion

5.3.1 Microbial analysis of thermally treated anaerobic activated sludge

The results from fluorescent in situ hybridization (FISH) technique are displayed in Fig. 8. Selected oligonucleotides probe in (Table 8) were used to detect major H_2 producing microorganisms in thermally treated mixed culture. The selected probes were successfully used in previous studies to target H_2 producers (Hwang et al., 2009b, Dębowski et al., 2014). Using the above probes, different strains of known H_2 producing bacteria, using the EUB mix specific for the bacterial domain, the *Gamma*– and *Betaproteobacteria* representing most fermentative bacteria in the phylum level of *Proteobacteria*, the *Enterobacteriacea* family and from *Clostridium* genus (chis150) representing most *Clostridium hystoliticum* were detected. Previous studies have shown the H_2 production potential of *Rahnella aquatilis* strain belonging to *Gammaproteobacteria* class (Dębowski et al., 2014). Many known H_2 producing bacteria belonging to the class of *Gamma* and *Beta* proteobacteria are characterized by their tolerance to harsh and wide temperatures, they ferment carbohydrates to produce acetate, propionate, H_2 and CO_2 (Im et al., 2012). Several species belonging to the *Enterobacteriaceae* family (*E. cloacae* and *E. aerogenes*) were reported to have the ability to degrade a wide variety of waste biomass such as lignocellulosic materials under adaptable environmental conditions (Jo et al., 2008, Shaterzadeh and Ataei, 2017).Some species of the genus *Clostridium* are known to be very efficient in H_2 production and was used in several studies as a pure or mixed culture for H_2 production (Pattra et al., 2008, Ortigueira et al., 2015, Reddy, 2016, Cabrol et al., 2017a). *Bacillus* spp. is a member of the phylum furmicutes, known to form spores, strains of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus subtilis* have been isolated from environmental samples and hydrogen producing reactors (Mishra et al., 2017a, Dhanasekar and Jonesh, 2018)



Figur 8. Fluorescence in situ hybridization (FISH) images showing hybridized cells by specific probes and DAPI stained cells: (a) and (b) most bacteria using EUB338, EUB338II, and EUB338III, (c) and (d) *Enterobacteriaceae* using EBAC1790, (e) *Gammaproteobacteria* using GAM42a, (f) *Betaproteobacteria* using BET42a, and (g) and (h) *Clostridium histolyticum* using Chis150

5.3.2 Isolation and Identification of hydrogen producing bacteria

A total of 10 colonies were selected on the overnight culture plate based on their difference in shape, color, size etc. (Table 16). The picked colonies were then distinguished and classified using Gram staining procedure and viewed under light microscope. After the DNA was extracted and screened for the hydrogenase gene, six isolates were amplified and were sequenced. Based on the phylogenetic analysis (Fig. 9), three of six isolates strains were closely related to *Bacillus cereus* and *Enterobacter cloacae*. None of strains have previously been investigated for fermentative H_2 production using *P. stratiotes*. However, are often reported from hydrogen producing reactors as the dominant microbes (Mishra et al., 2017a, Sołowski et al., 2018). The strains were further investigated as pure-cultures for H_2 production via dark-fermentation, in which the cultures were operated at the optimum condition of pH 5.5, time 8 h, S/X 1 gCOD/gVSS, and temperature 25°C. The results from this study are discussed in detail below.

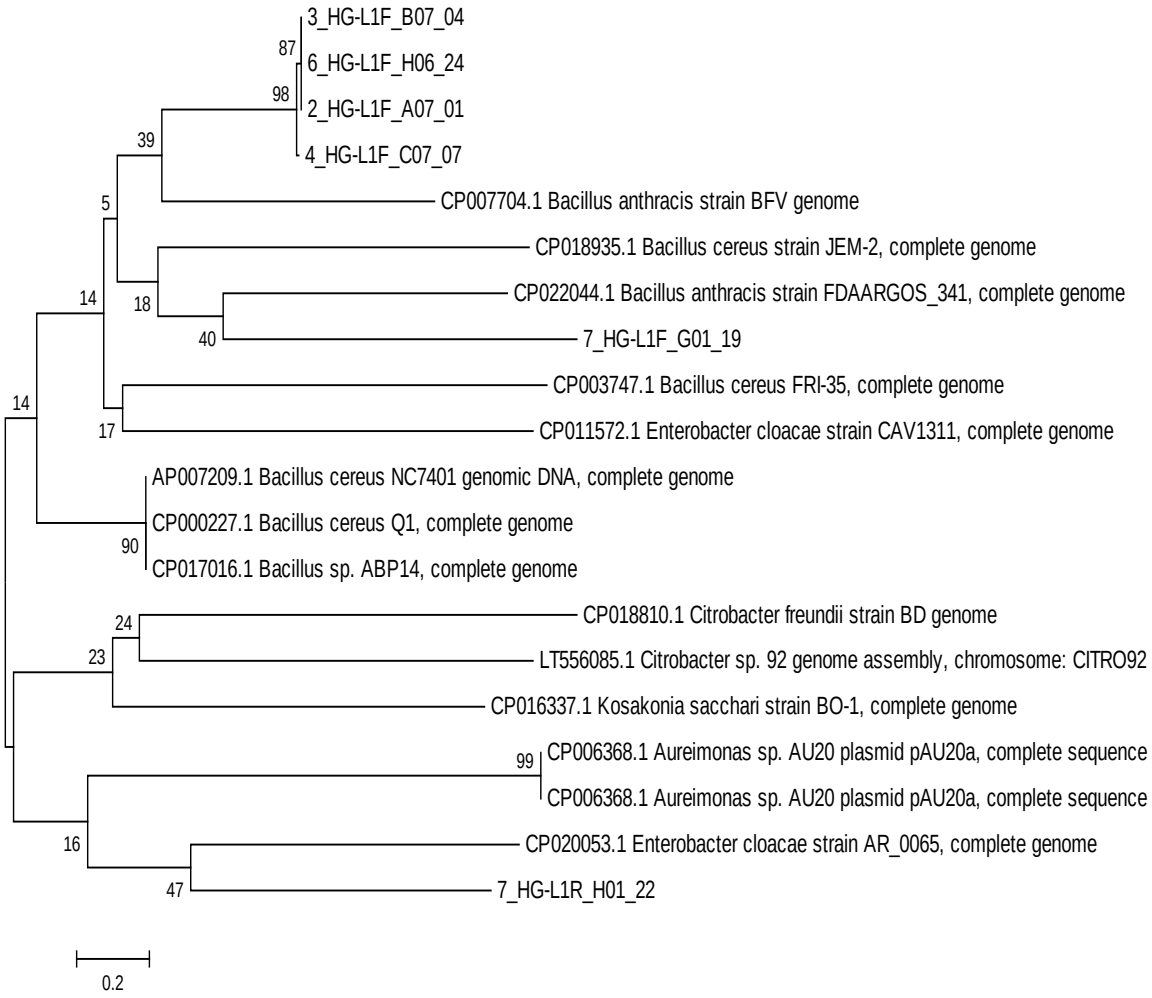


Figure 9. Neighbor-joining tree showing phylogenetic position of biohydrogen-producing strains based on 16S rRNA sequences

5.3.3 Hydrogen production potential of bacterial isolates

a) *Bacillus cereus* 3_HG-L1F

The isolate 3_HG-L1F, showed similarity with *Bacillus cereus* both morphologically and phylogenetically (figure 9, 10a, 10b). The cells are rod-shaped that tended to form irregular colonies (grape-like clusters). The spores of *Bacillus cereus* can resist the thermal treatment (Setlow, 2006, Liu and Wang, 2012). The isolate achieved a HY of 2.21 mol H_2/mol glucose, equivalent to 3.15 mg H_2/gDW (Table 16) under optimized conditions. Previous studies have shown that the *Bacillus cereus* strain are able to utilize various bio-resources and bio wastes and generate H_2 (Zhang et al., 2014, ali shah et al., 2016, Prakash et al., 2018). Similarly, Patel et al. (2011) achieved HYs of 0.96, to 1.92 mol H_2/mol glucose using different strains of *Bacillus cereus*.

b) *Enterobacter cloacae* 7_HG-L1F

Additional isolated bacterial species, namely 7_HG-L1F, was determined to belong to *Enterobacter cloacae*. *Enterobacter* is Gram–negative and facultative anaerobes recognized as small rod-shaped bacteria (Fig. 10d). In this study, the isolate achieved a HY of 1.97 mol H_2/mol glucose, corresponding to 2.18 mg H_2/gDW (Table 16). The satisfactory HY could be due to the effective hydrolytic enzyme activity of *Enterobacter cloacae*. Sun et al. (2015) attempted to isolate *Enterobacter cloacae* from sugar refinery sludge for H_2 production and attained H_2 yield 707 mL H_2 per litre medium. Harun et al. (2012) investigated the isolation of *Enterobacter cloacae* KBH3 from termite guts, their study demonstrated that *Enterobacter cloacae* KBH3 was able to utilize carbon and generate H_2 within a wide range of pH (4.5–8) and temperature (28–40°C), with a satisfactory HY of 1.8 mol H_2/mol glucose.

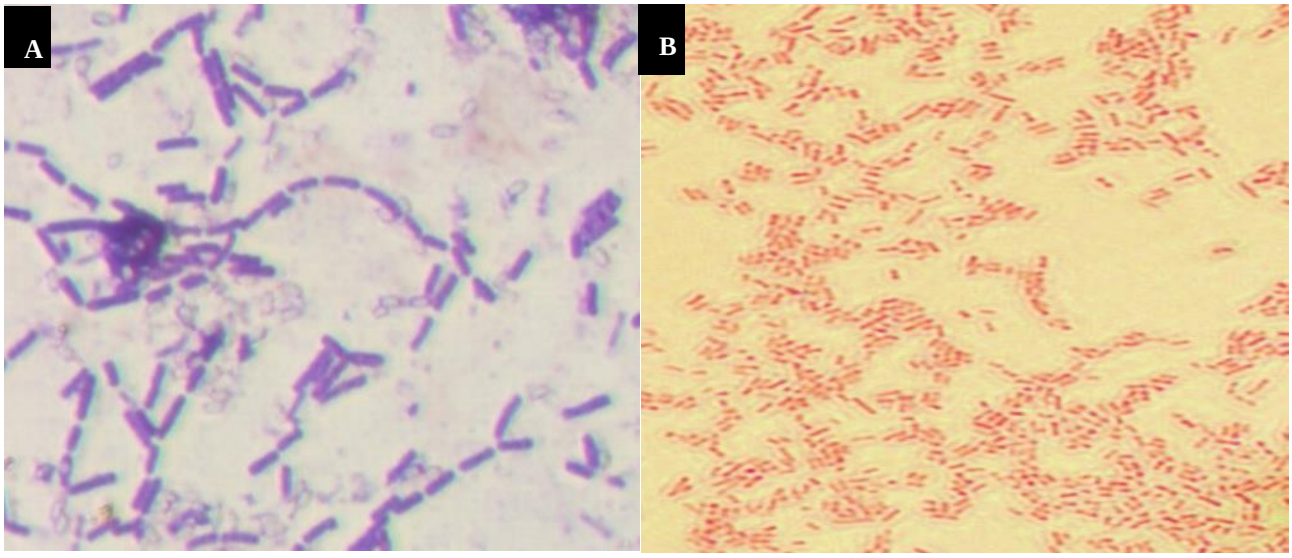


Figure 10. Gram stain images under 100x oil immersion objective lens, 1000x magnification a) *Bacillus cereus* and b) *Enterobacter cloacae*

5.3.4 Comparison between mixed culture and pure culture

It was demonstrated that pure-isolates similar to *Bacillus cereus* and *Enterobacter cloacae* were efficient for H_2 production from *Pistia stratiotes* via dark fermentation. Similar findings have been reported in previous studies using various types of substrates (Bao et al., 2012, Harun et al., 2012, Song et al., 2013, Mishra et al., 2015, Mohanraj et al., 2016). However, the mixed culture attained a higher HY of 2.46 mol H_2/mol glucose as compared to that of individual strains (HY: 1.10 – 2.21 mol H_2/mol glucose) under similar fermentation conditions. This result could be linked to the thermal pre-treatment of sludge that prevented the dominance of H_2 consumers while maintaining the activity of the H_2 producing bacterial consortia. In addition, the diversity of H_2 producers in mixed cultures could enhance the organics utilization and avoid substrate inhibition Moreover, it is difficult to isolate and identify all the bacteria involved in H_2 production using culture dependent techniques. Most of them are non-cultivable and hence difficult to isolate. Presence of higher percentage of non-cultivable H_2 producers in mixed culture could be a reason for higher HY in thermally treated mixed culture inoculum. Fluorescence in situ hybridization results also demonstrated that the microbial community

in the mixed culture comprised of multiple H_2 producing bacteria such *Enterobacteriaceae*, *Gammaproteobacteria*, *Betaproteobacteria*, and *Clostridium histolyticum* which could contribute to HY compared to single culture additionally *Clostridum* is known to have higher H_2 production potential compared to *Bacillus* spp. and *Enterobacter* spp. Cabrol et al., (2017) also reported that a wide range of phylogenetically different hydrogen producing microbial diversity in mixed microbial consortia. Most of them are non–culturable and hence are difficult to isolate in pure culture.

A mixed culture of H_2 producers is generally favourable over pure culture due to wider feedstock choice, practicability for environmental engineering applications, and economic benefits (Wang and Wan, 2009). Similarly, Bao et al. (2012) demonstrated that the combination of two bacterial species belonging to *Bacillus* and *Brevumdimonas* enhanced the HY to 1.04 mol H_2/mol glucose using starch, being twice that obtained from pure-cultures. Their study confirmed that fermentation using a single bacterium species could attain a limited efficiency of H_2 production. Moreover, Mishra et al. (2015) found that HY obtained from acidogenic mixed consortia (156 mmol H_2/L) was higher than that obtained using synthetic co-culture (117.4 mmol H_2/L) and pure-culture (98.2 mmol H_2/L). Their study indicated that the existence of *Clostridium* sp. containing Fe–Fe hydrogenase in the mixed culture could produce a high HY of 4 mol H_2/mol glucose (Mishra et al., 2015). On the other side, Antonopoulou et al. (2007) reported that bioH_2 production from sweet sorghum using pure-cultures of *Ruminococcus albus* attained a higher HY of 2.6 mol H_2/mol glucose compared to that of mixed-culture (0.86 mol H_2/mol glucose). This result could be linked to the fulfilment of optimum operating conditions for each distinct strain. In addition, the produced H_2 by mixed culture could be readily reduced by H_2 consuming bacteria, such as homoacetogens and methanogens (if no appropriate sludge treatment was performed). Hence, it is recommended to complement the H_2 producing abilities of various bacterial strains as mixed cultures. However, further investigations are in progress to

understand the metabolic and biochemical pathways as well as genetic engineering for H_2 production in each pure culture.

Table 16. Morphology, phenotypic characterization and substrate utilization of the isolates recovered from the mesophilic mixed culture sludge plus *P. stratiotes*

Isolate	**3_HG-L1F**	**7_HG-L1F**
Cell shape	Rod-shaped (chained)	Small rod-shaped (clusters/dispersed)
Cell size	> 5 μm	> 5 μm
Gram stain	Gram (+)	Gram (-)
Colony	Large irregular round	Cauliflower type
HY (mol-H_2/mol-glucose)	2.21	1.97
HY (mg-H_2/g-dw)	3.15	2.81
HP (mL-H_2/L)	411	365
Closest relative	*Bacillus cereus*	*Enterobacter cloacae*
Similarity (%)	99%	99%

5.4 Conclusions

- Fluorescence in situ hybridization results demonstrated that the microbial community in the mixed-culture comprised of H_2 producing bacteria, viz., *Enterobacteriaceae*, and *Clostridium histolyticum*.
- Three H_2 producing strains were successfully isolated from enriched mixed culture reactor and were identified to belong to *Bacillus cereus* and *Enterobacter cloacae*. All the three isolates displayed the hydrogen production potential achieving HYs of 2.21, 1.10, and 1.97 mol H_2/mol glucose, respectively under similar fermentation condition.
- The maximum H_2 yield of 2.48 mol H_2/mol glucose was obtained from the thermal treated mixed culture in comparison to pure cultures.

CHAPTER VI

COST ESTIMATION FOR THE PRODUCTION OF HYDROGEN FROM *PISTIA STRATIOTES* THROUGH DARK FERMENTATION PROCESS USING THERMALLY TREATED SLUDGE AS AN INOCULUM

6.1 Introduction

The world's energy market is currently dominated by fossil fuels such as natural gas, coal and crude oil (Li and Sun, 2018). South Africa (SA) generates its energy needs from coal (35%), crude oil (50%) and gas (5%) (SAPIA, 2018). Statistics reports indicate that 80% of SA's crude oil is imported, as a result, the country has shifted from being a net export market to being a net import market (Fin24, 2014). Moreover, since 2016 the price of oil has risen from $30 a barrel up to $80 a barrel due to depletion of natural resources (SAGovNew, 2018). In addition, a further increase in fuel cost is projected in the near future (Narbel and Hansen, 2014). Therefore, sustainable and renewable energy sources are required as a substitute for fossil fuels.

The overall cost of hydrogen production however, can be influenced by several factors including the type of culture and its production rate, the type of reactor configuration, electricity, substrate, labour as well as general chemical and consumables requirements (Khamis and Malshe, 2010, El-Emam et al., 2015). Studies indicated that direct biophotolysis process can produce hydrogen at a rate of 1.68 mmole H_2/litre-day, the indirect biophotolysis process produces H_2 at a rate of 8.64 mmoles H_2/litre-day, photo fermentation produces hydrogen at a rate of 3.672 moles /litre-day and dark fermentation process produce hydrogen at a rate of 2.904 moles/litre-day (when the same rate is constant for 24hours) (Sathyaprakasan and Kannan, 2015).

The bioreactor configuration employed for direct biophotolysis is generally a tubular reactor (Dasgupta et al., 2010), whereas indirect biophotolysis require an open photo-bioreactor, micro-algae in a pond which requires about 8,490 acres of land (27000 ponds) for optimal hydrogen production. Photo fermentation may also employ an open photo-bioreactor system however, due to the additional fermentation step of acetate break down, an additional land of 2120 acre is required for layout (Caia et al., 2018). Indirect biophotolysis is a continuous process, where its requires three stages (three reactor design), 1) open ponds containing algae that produces glucose from carbon dioxide and water, 2) the produced glucose is thereafter passed to the dark fermentation reactor to produce hydrogen and metabolic products (acetate, butyrate) which are further 3) broken down in a photo fermenter to produce hydrogen (Sathyaprakasan and Kannan, 2015). The operational cost estimated based on the land required for each process state that the indirect biophotolysis is more expensive compared to other two processes, as it requires a large land for continuous reactor, more chemicals and consumables, electricity and labour to operate the different stages, thereby increasing the capital (Nikolaidis and Poullikkas, 2017, Chia et al., 2018).

Dark fermentation possesses several advantages including satisfactory H_2 production rates, reduction of wastes and pollution, and low energy requirements (Kumar et al., 2018b). Dark fermentation requires simple bioreactor that covers approximately 1.700 acre of space (Sathyaprakasan and Kannan, 2015). This process utilises waste substrate such as agricultural waste (sorghum bagasse and crop residues), food waste, aquatic weeds, and animal manure to drive the process (Łukajtis et al., 2018). These substrates are cost effective and contains high carbon sources thus reduces the cost overall operational costs (Montecchio et al., 2018).

Several studies have been conducted to improve the methods for hydrogen yield through dark fermentation from different substrate (Nikolaidis and Poullikkas, 2017, Yang and Wang, 2018). However, the information on the economic analysis of biohydrogen production from different biomass is scarce. Furthermore, the information on cost analysis part of H_2 production from aquatic weeds is limited. Therefore, this objective focused on the cost estimation of biohydrogen production from aquatic weed (*P. stratiotes*) using thermally treated sludge as an inoculum. The common factors affecting the commercial viability of dark fermentative hydrogen production were assessed.

6.2 Methods

6.2.1 Cost estimation

The economic analysis of production of biohydrogen using dark fermentation from aquatic weed *P. stratiotes* was analysed based on the data generated from optimisation study in chapter four and the experimental setup in section 4.2.3. The total cost (TC) of a full-scale batch reactor was estimated by considering the amortization cost of the investment (AC) and operating cost (OC) based on a daily hydrolysate of 4.0 m^3 (i.e., 265 kg-DW of *Pistia stratiotes*), the fermenter volume could be 2.7 m^3.

6.2.1.1 Amortization cost (AC)

The amortization cost was calculated based on the construction items and the required mechanical and electrical facilities (Nasr et al., 2013). The treatment unit capacity (V_c, in m^3) was computed based on the ratio of the working hours per day (t_w: assuming 16 h/day) to the treatment time in each batch (t_t: assuming 8 h), as seen in Eq. (31) (Alalm et al., 2015).

$$V_c = \frac{V_t}{D} \times \frac{t_t}{t_w} \tag{31}$$

where, V_t is the total treated volume on a yearly basis (m^3/year), and D is the number of operating days (i.e., 300 d/year). A 10% of V_c was added as a headspace for the H_2 gas collection. The amortization cost of investment per cubic meter of wastewater was calculated by Eq. (32) (Alalm et al., 2017).

$$AC = \frac{C_p \times V_c}{L \times V_t} \tag{32}$$

where, C_p is the cost per cubic meter of the fermentation unit (C_p: assuming 10000 \$/m^3 based on quotes from local suppliers and contractors), and L is the lifecycle of the construction facilities (L: assuming 10 years). The footprint item was estimated as 0.16 \$/m^3 (equivalent to 8.5% of AC). The calculated percentages in Table 18 were approximately similar to those obtained in a previous study by Nasr et al., (2015). Based on a daily hydrolysate of 4.0 m^3 (i.e., 265 kg-DW of *Pistia stratiotes*), the fermenter volume could be 2.7 m^3 resulting in AC of 1.83 \$/m^3 (equivalent to 36% of the TC). This amount of feed would provide a robust reactor design and simple construction with few mechanical resistance materials.

6.2.1.2 Operation cost (OC)

The operation cost included the inoculum treatment procedure, substrate hydrolysis step, chemicals supply, and energy consumption. The labor expenses were not involved in this study for simplification because the fermentation process does not require intensive manpower. The unit price (P_i, in \$/kg) of each item was obtained based on a cost survey from local treatment plants and administrative databases in the period of 2015 – 2017. The P_i values were 0.6 \$/kg for the inoculum including collection, transportation, and thermal treatment, 0.6 \$/kg for substrate hydrolysis, and 0.5 \$/kg for chemicals (e.g., supplements and pH adjustment). The costs of the aforementioned items were calculated from the concentration (C_i, in kg/m^3) multiplied by the unit price (P_i, in \$/kg).

The energy cost (EC, in \$/m^3) was calculated by Eq. (33) based on the power required for mixing, heating, and equipment operation.

$$EC = \frac{E \times P_E \times t_w \times D}{V_t} \quad (33)$$

where, E is the consumed power (kW), P_E is the unit price of electricity cost (i.e., 0.26 $/kW/h). Maintenance cost was assumed as 2% of the amortization cost (Alalm et al., 2017). Hence, the total operating cost was calculated by Eq. (34).

$$OC = (C_i \times P_i) + EC + \frac{2}{100} AC \tag{34}$$

6.3 Results and discussion

The economic analysis of dark fermentation for biohydrogen production from *P. stratiotes* hydrolysate is listed in Table 17 (currency in USD). The results indicated that the electricity consumption was the highest OC item, providing 1.88 $/m^3 (i.e., 57.6% of OC). The electricity required for device operation was equivalent to 28.9 kW·h/day. Under this condition, the OC was estimated as 3.27 $/m^3 (i.e., OC = 64.1% of TC).

The OC estimated in this study was higher than the AC which could be related to AC being a once off expenditure and OC as a recurring expense required for production of hydrogen, it is expected to be higher. The findings also revealed that the Total Cost (TC) including AC and OC was 5.10 $/m^3, equivalent to 0.08 $/kg-DW of *P. stratiotes* and 22.8 $/kg-$H_2$ produced. The cost estimated is cheaper compared to other studies (Ljunggren and Zacchi, 2010, Ljunggren et al., 2011, Sathyaprakasan and Kannan, 2015), indicating that the process used in this study is cost efficient. The inexpensive substrate and anaerobic activated sludge used in this study could be the contributing factor towards the low cost. The optimum temperature of 25°C for H_2 production in this study could also be the contributing factor in the reduction of cost, because higher temperatures would have required higher energy consumption (Chovau et al., 2013, Keipi et al., 2018). With electricity consumption being the highest contributing item in the OC, if a cheaper source of electricity could be used for this process the total cost could be reduced further. A previous study evaluated economic analysis of hydrogen production from CH_4 thermal decomposion and water electrolysis and concluded that the main factors that affected the economic feasibility was the electricity cost, carbon value and natural gas thus economic feasibility highly depend on a renewable feedstock and electricity (Keipi et al., 2018).

A close value of 18.7 \$/kg-H_2 via dark fermentation using algae cultures was reported in a study by Sharma and Kaushik (2010), this cost is higher than the cost estimated from this study probably due to the choice of culture (pure culture) used. Biohydrogen production using pure cultures requires a lot stable conditions resulting in higher costs. Moreover, Ljunggren and Zacchi (2011) found a H_2 production cost of 24.6 \$/kg-H_2 via simultaneous steps of pre–treatment, dark fermentation, photo–fermentation, and gas purification using potato steam peels as a substrate. However, a high value of 62.9 \$/kg-H_2 was obtained in a study by Ljunggren et al., (2011) through a combined dark and photo fermentation process using barley straw as feedstock. The high costs estimated here could be due to the photo–fermentation process, gas purification and the substrate being used in their study.

Table 17. Summary of economic analysis (currency in USD) for H_2 fermentation from *P. Stratiotes*

Item	Amount (\$/m^3)	Percentage (%)
Amortization cost (AC)		
Construction	0.62	33.6
Mechanical equipment	0.81	44.2
Footprint	0.16	8.5
Others (e.g., Electrical wiring, piping, fittings)	0.25	13.7
Sum of capital cost	1.83 (0.03 \$/kg-DW)	100
Operational cost (OC)		
Sludge handling and processing	0.30	9.2
Substrate hydrolysis	0.90	27.5
Chemical supply	0.15	4.6
Electricity consumption	1.88	57.6
Maintenance cost	0.04	1.1
Sum of operational cost	3.27 (0.05 \$/kg-DW)	100
Total cost (TC)		
TC = AC + OC	5.10 (0.08 \$/kg-DW)	

6.4 Conclusions

The total cost of the fermentation system including the amortization cost of investment and operating cost was 0.08 $/kg-dry weight (22.8 $/kg-H2 produced). Based on the cost estimation, this method of biohydrogen production from aquatic weed (*P. stratiotes*) through dark fermentation is relatively feasible compared to other methods. However, factors such as inoculum type, feedstock and reactor configuration could change, thus affecting the overall cost.

CHAPTER VII:

GENERAL CONCLUSIONS AND RECOMMENDATIONS

7.1 Summary and conclusions

The foremost objective of the study was to evaluate biohydrogen production potential of aquatic weed *Pistia stratiotes* through dark fermentation to offer an efficient and environmentally friendly approach for H_2 production. Previously *P. stratiotes* has mostly been investigated for ethanol and methane production. Thus far beside this study, only one study has previously investigated the utilization of the aquatic weed *P. stratiotes*, for biohydrogen production. However, via a photo fermentation process using a pure culture (*Rhodopseudomonas palustris 42OL and Rhodopseudomonas palustris CGA676*) as an inoculum (Corneli et al., 2017). Compared to this study, an anaerobic activated sludge (mixed culture) was used as an inoculum via the dark fermentation processes.

To accomplish improved H_2 yield and rate, the biomass was first characterized for suitability as a substrate and was thereafter subjected to different hydrolysis methods to obtain high concentration of reducing sugars and digestibility. The operational conditions for optimum H_2 production were optimized via dark fermentation using the hydrolysate and a thermally anaerobic activated sludge as an inoculum. Microbial analyses using FISH, PCR, and qPCR confirmed and correlated the H_2 productivity results. Lastly the effect of the type of inoculum on H_2 production was studied by isolation and identification techniques.

The major conclusion of this study includes:

- The suitability of the substrate (*Pistia stratiotes*) for fermentation was indicated by the C: N of 25.7, low lignin (3.94% and 1.14%) percentage and high cellulose (37.83%) percentage,

indicating possible easy solubility of lignin that is adequate to generate fermentable sugars with optimum pre–treatments.

- The substrate was subjected to different pre–treatment methods i.e. physical, chemical and physical–chemical methods to improve its susceptibility to hydrolysis and for production of maximum fermentable sugars. Among the pre–treatment employed, the hydrolysis pre–treatment process of *P. stratiotes* for 30 minutes achieved the maximum respective sugar yield of 139.8 mg/g after steam at 121°C pre–treatment of 2.5% H_2SO_4. The sugar yield was greatly improved (139.8 mg/g) under optimized pretreatment conditions when compared to the untreated biomass (20.9 mg/g).
- Scanning electron microscopic analysis validated the effectiveness of steam pre–treatment of 2.5% H_2SO_4 at 121°C in hydrolysis of *P. stratiotes*. The results showed the correlation between the surface destruction of the biomass and the maximum sugar yield obtained.
- Thermal pre–treatment (100°C, 45 min) of anaerobic sludge was effective in eliminating the H_2 consuming microorganisms whilst promoting the enrichment of spore–forming H_2 producing bacteria, which was confirmed by using FISH with specific probes.
- Biohydrogen fermentation was estimated at different experimental factors, viz., pH, fermentation time, culture temperature, and substrate–to–biomass (S/X) ratio to obtain optimum levels. The highest hydrogen yield of 2.46 mol-H_2/mol-glucose (3.51 mg-H_2/g-DW) was obtained at optimum operational conditions of pH 5.5, HRT 8 h, temperature 25°C, and S/X 1.
- Electron-equivalent balance of 92 – 98% and COD mass balance of 92 – 96%, indicated the successful transfer of organic carbon to the production of H_2 gas and metabolic intermediates thus the reliability of the aforementioned results. A quadratic model was developed to predict the HY using the experimental factors as inputs (R^2: 0.928). The solution pH provided

positive-linear and negative-quadratic effects on the HY, whereas the plot of HY against S/X showed a negative–linear curve.

- The qPCR and PCR results indicated a positive correlation between the abundance of H_2 producing bacteria and H_2 production potential based on the hydrogenase gene copy number/nanogram of genomic DNA. The hydrogenase gene exhibited the highest activity at pH of 5.5 with 2.53×10^4 copies/ng-DNA compared to low pH range of 4.5 (6.95 × 103 copies/ng-DNA) and highest pH range 8.5 (7.77 × 103 copies/ng-DNA).
- Two H_2 producing strains were isolated from enriched mixed culture reactor operated under the optimum environmental conditions. The bacterial species were identified to belong to *Bacillus cereus* and *Enterobacter cloacae.* All the three isolates displayed the H_2 production HYs of 2.21, 1.10, and 1.97 mol H_2/mol glucose, respectively under similar fermentation condition. The maximum H_2 yield of 2.48 mol H_2/mol was obtained from the thermally treated mixed culture in comparison to pure isolates.
- The total cost of the fermentation system including the amortization cost of investment and operating cost was 0.08 $/kg-dry weight (22.8 $/kg-H2 produced). Based on the cost estimation, this method of biohydrogen production from aquatic weed (*P. stratiotes*) through dark fermentation is relatively feasible compared to other methods.

7.2 Recommendations

- This study focused on batch operation and produced satisfactory hydrogen yield, however commercial feasibility future studies are recommended especially with larger scale and continuous operation of dark fermentation using *Pistia stratiotes.*
- Isolation and identification of additional indigenous H_2 producing strains from the optimum fermentation and continuous reactor is also recommended with a focus on the whole genome

sequence/genetic diversity to obtain possible new strains with the ability to produce H_2 since this study only targeted specific 16S rRNA sequences.

- Evaluation of combined aquatic weeds/co-digestion of different aquatic weeds to potentially increase the amount of fermentable sugars is also recommended.
- Additional economic and environmental values for the use of aquatic weeds in the production of hydrogen is recommended. This will provide a more rigorous cost benefit analysis for biohydrogen production using this substrate.

REFERENCES

ABDALLA, A. M., HOSSAIN, S., NISFINDY, O. B., AZAD, A. T., DAWOOD, M. & AZAD, A. K. 2018. Hydrogen production, storage, transportation and key challenges with applications: A review. *Energy Conversion and Management,* 165**,** 602-627.

ADEKUNLE, K. F. & OKOLIE, J. A. 2015. A Review of Biochemical Process of Anaerobic Digestion. *Advances in Bioscience and Biotechnology,* 06**,** 205-212.

AKANKSHA, K., PRASAD, A., SUKUMARAN, R. K., NAMPOOTHIRI, M. K., PANDEY, A., RAO, S. & BINOD, P. 2014. Dilute acid pretreatment and enzymatic hydrolysis of sorghum biomass for sugar recovery—a statistical approach.

AKROUM-AMROUCHE, D., ABDI, N., LOUNICI, H. & MAMERI, N. 2013. Biohydrogen production by dark and photo-fermentation processes. 499-503.

AKUTSU, Y., LI, Y.-Y., HARADA, H. & YU, H.-Q. 2009. Effects of temperature and substrate concentration on biological hydrogen production from starch. *International Journal of Hydrogen Energy,* 34**,** 2558-2566.

ALALM, M. G., TAWFIK, A. & OOKAWARA, S. 2015. Degradation of four pharmaceuticals by solar photo-Fenton process: kinetics and costs estimation. *Journal of Environmental Chemical Engineering,* 3**,** 46-51.

ALALM, M. G., TAWFIK, A. & OOKAWARA, S. 2017. Investigation of optimum conditions and costs estimation for degradation of phenol by solar photo-Fenton process. *Applied Water Science,* 7**,** 375-382.

ALBALASMEH, A. A., BERHE, A. A. & GHEZZEHEI, T. A. 2013. A new method for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometry. *Carbohydr Polym,* 97**,** 253-61.

ALI SHAH, F., MAHMOOD, Q., MAROOF SHAH, M., PERVEZ, A. & AHMAD ASAD, S. 2014. Microbial Ecology of Anaerobic Digesters: The Key Players of Anaerobiosis. *The Scientific World Journal,* 2014**,** 183752.

ALI SHAH, T., LORENZO, F., ALIBARDI, L., CAGNIN, L., SANDON, A., COSSU, R., SERGIO, C. & BASAGLIA, M. 2016. *Bacillus sp. strains to produce bio-hydrogen from the organic fraction of municipal solid waste*.

AMIDZADEH, Z., BEHBAHANI, A. B., ERFANI, N., SHARIFZADEH, S., RANJBARAN, R., MOEZI, L., ABOUALIZADEH, F., OKHOVAT, M. A., ALAVI, P. & AZARPIRA, N. 2014. Assessment of Different Permeabilization Methods of Minimizing Damage to the Adherent Cells for Detection of Intracellular RNA by Flow Cytometry. *Avicenna Journal of Medical Biotechnology*, 6**,** 38-46.

AMIN, MEHDI&, M., BIJAN BINA1, E. T., FATEHIZADEH, A. & GHASEMIAN, M. 2016. Stoichiometry evaluation of biohydrogen production from various carbohydrates. *# Springer***,** 20915–20921.

AMIN, F. R., KHALID, H., ZHANG, H., RAHMAN, S. U., ZHANG, R., LIU, G. & CHEN, C. 2017. Pretreatment methods of lignocellulosic biomass for anaerobic digestion. *AMB Express*, 7**,** 72.

ANAEROB, M. K. C., PATTRA, S. & SITTIJUNDA, S. 2017. Biohydrogen productions from hydrolysate of water hyacinth stem (Eichhornia crassipes) using anaerobic mixed cultures. *Sains Malaysiana*, 46**,** 51-58.

ANTONOPOULOU, G., NTAIKOU, I., GAVALA, H., SKIADAS, I., ANGELOPOULOS, K. & LYBERATOS, G. 2007. Biohydrogen production from sweet sorghum biomass using mixed acidogenic cultures and pure cultures of Ruminococcus albus. *Global Nest J*, 9**,** 144-151.

ARGUN, H. & DAO, S. 2016. Hydrogen gas production from waste peach pulp by dark fermentation and electrohydrolysis. *International Journal of Hydrogen Energy*, 41**,** 11568-11576.

ARGUN, H., GOKFILIZ, P. & KARAPINAR, I. 2017. Biohydrogen Production Potential of Different Biomass Sources. 11-48.

ASLAM, M., AHMAD, R., YASIN, M., KHAN, A. L., SHAHID, M. K., HOSSAIN, S., KHAN, Z., JAMIL, F., RAFIQ, S., BILAD, M. R., KIM, J. & KUMAR, G. 2018. Anaerobic membrane bioreactors for biohydrogen production: Recent developments, challenges and perspectives. *Bioresource Technology*, 269**,** 452-464.

ATEIA, M., NASR, M., IKEDA, A., OKADA, H., FUJII, M., NATSUIKE, M. & YOSHIMURA, C. 2016. Nonlinear relationship of near-bed velocity and growth of riverbed periphyton. *Water*, 8**,** 461.

AWOLUSI, M, N., S, K. & F, B. 2016. Principal component analysis for interaction of nitrifiers and wastewater environments at a full-scale activated sludge plant. *Durban university of technology*.

AZMAN, S., KHADEM, A. F., VAN LIER, J. B., ZEEMAN, G. & PLUGGE, C. M. 2015. Presence and Role of Anaerobic Hydrolytic Microbes in Conversion of Lignocellulosic Biomass for Biogas Production. *Critical Reviews in Environmental Science and Technology,* 45**,** 2523-2564.

BAJPAI, P. 2017. Anaerobic Technology in Pulp and Paper Industry. *SpringerBriefs in Applied Sciences and Technology*. DOI 10.1007/978-981-10-4130-3–2

BALAT, M. 2008. Possible Methods for Hydrogen Production. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects,* 31**,** 39-50.

BANU, J. R., YUKESH KANNAH, R., DINESH KUMAR, M., GUNASEKARAN, M., SIVAGURUNATHAN, P., PARK, J.-H. & KUMAR, G. 2018. Recent advances on biogranules formation in dark hydrogen fermentation system: Mechanism of formation and microbial characteristics. *Bioresource Technology,* 268**,** 787-796.

BAO, M., SU, H. & TAN, T. 2012. Biohydrogen Production by Dark Fermentation of Starch Using Mixed Bacterial Cultures of Bacillus sp and Brevumdimonas sp. *Energy & Fuels,* 26**,** 5872-5878.

BARAKAT, A., MAYER-LAIGLE, C., SOLHY, A., ARANCON, R. A. D., DE VRIES, H. & LUQUE, R. 2014. Mechanical pretreatments of lignocellulosic biomass: towards facile and environmentally sound technologies for biofuels production. *RSC Advances,* 4**,** 48109-48127.

BARAKAT, A., MONLAU, F., SOLHY, A. & CARRERE, H. 2015. Mechanical dissociation and fragmentation of lignocellulosic biomass: Effect of initial moisture, biochemical and structural proprieties on energy requirement. *Applied Energy,* 142**,** 240-246.

BARUA, V. B. & KALAMDHAD, A. S. 2018. Anaerobic biodegradability test of water hyacinth after microbial pretreatment to optimise the ideal F/M ratio. *Fuel,* 217**,** 91-97.

BHARATHIRAJA, B., SUDHARSANAA, T., BHARGHAVI, A., JAYAMUTHUNAGAI, J. & PRAVEENKUMAR, R. 2016. Biohydrogen and Biogas – An overview on feedstocks and enhancement process. *Fuel,* 185**,** 810-828.

BHATTACHARYA, A., SADHUKHAN, A. K., GANGULY, A. & CHATTERJEE, P. K. 2016. Dilute Acid Induced Changes on Microscopic and Tomographic Structure of Water Hyacinth [Eichhornia

Crassipes (Mart.) Solms] Biomass during Bioconversion Process to Xylitol. *Indian Journal of Science and Technology*, 9.

BUNDHOO, M. A. Z., MOHEE, R. & HASSAN, M. A. 2015. Effects of pre-treatment technologies on dark fermentative biohydrogen production: A review. *Journal of Environmental Management*, 157**,** 20-48.

BUNDHOO, M. Z. & MOHEE, R. 2016. Inhibition of dark fermentative bio-hydrogen production: a review. *International Journal of Hydrogen Energy*, 41**,** 6713-6733.

BUNDHOO, Z. M. A. 2017. Coupling dark fermentation with biochemical or bioelectrochemical systems for enhanced bio-energy production: A review. *International Journal of Hydrogen Energy*, 42**,** 26667-26686.

CABROL, L., MARONE, A., TAPIA-VENEGAS, E., STEYER, J.-P., RUIZ-FILIPPI, G. & TRABLY, E. 2017a. Microbial ecology of fermentative hydrogen producing bioprocesses: useful insights for driving the ecosystem function. *FEMS Microbiology Reviews*, 41**,** 158-181.

CABROL, L., MARONE, A., TAPIA-VENEGAS, E., STEYER, J. P., RUIZ-FILIPPI, G. & TRABLY, E. 2017b. Microbial ecology of fermentative hydrogen producing bioprocesses: useful insights for driving the ecosystem function. *FEMS Microbiol Rev*, 41**,** 158-181.

CAIA, M., BERNARD, O. & BÉCHET, Q. 2018. Optimizing CO2 transfer in algal open ponds. *Algal Research*, 35**,** 530-538.

CAO, G.-L., ZHAO, L., WANG, A.-J., WANG, Z.-Y. & REN, N.-Q. 2014. Single-step bioconversion of lignocellulose to hydrogen using novel moderately thermophilic bacteria. *Biotechnology for biofuels*, 7**,** 82.

CAPPAI, G., DE GIOANNIS, G., MUNTONI, A., POLETTINI, A., POMI, R. & SPIGA, D. Effect of inoculum to substrate ratio (ISR) on hydrogen production through dark fermentation of food waste. 2015.

CARDOSO, V., ROMÃO, B. B., SILVA, F. T., SANTOS, J. G., BATISTA, F. R. & FERREIRA, J. S. 2014. Hydrogen production by dark fermentation. *Chem Eng Trans*, 38**,** 481-486.

CHAGANTI, S. R., LALMAN, J. A. & HEATH, D. D. 2012. 16S rRNA gene based analysis of the microbial diversity and hydrogen production in three mixed anaerobic cultures. *International Journal of Hydrogen Energy*, 37**,** 9002-9017.

CHAITANYA, N., SATISH KUMAR, B., HIMABINDU, V., LAKSHMINARASU, M. & VISHWANADHAM, M. 2018. Strategies for enhancement of bio-hydrogen production using mixed cultures from starch effluent as substrate. *Biofuels,* 9**,** 341-352.

CHAMIER, J., SCHACHTSCHNEIDER, K., LE MAITRE, D. C., ASHTON, P. J. & VAN WILGEN, B. W. 2012. Impacts of invasive alien plants on water quality, with particular emphasis on South Africa. *Water SA,* 38.

CHANDRASEKHAR, K., LEE, Y. J. & LEE, D. W. 2015. Biohydrogen production: strategies to improve process efficiency through microbial routes. *Int J Mol Sci,* 16**,** 8266-93.

CHAUDHARY, A., THAKUR, V., QURAISHI, A. & JADHAV, S. 2015. Isolation and characterization of biohydrogen producing bacteria from rice bran with optimization of different parameters.

CHEN, C.-Y., YANG, M.-H., YEH, K.-L., LIU, C.-H. & CHANG, J.-S. 2008. Biohydrogen production using sequential two-stage dark and photo fermentation processes. *International Journal of Hydrogen Energy,* 33**,** 4755-4762.

CHEN, H., LIU, J., CHANG, X., CHEN, D., XUE, Y., LIU, P., LIN, H. & HAN, S. 2017. A review on the pretreatment of lignocellulose for high-value chemicals. *Fuel Processing Technology,* 160**,** 196-206.

CHEN, K.-Y., ZHENG, Y. & CHENG, Y.-S. 2015. Integrated alkali pretreatment and preservation of wet lettuce (Pistia stratiotes) by lactic acid bacteria for fermentable sugar production. *Biomass and Bioenergy,* 81**,** 249-255.

CHIA, S. R., CHEW, K. W., SHOW, P. L., YAP, Y. J., ONG, H. C., LING, T. C. & CHANG, J.-S. 2018. Analysis of Economic and Environmental Aspects of Microalgae Biorefinery for Biofuels Production: A Review. *Biotechnology Journal,* 13**,** 1700618.

CHOVAU, S., DEGRAUWE, D. & VAN DER BRUGGEN, B. 2013. Critical analysis of techno-economic estimates for the production cost of lignocellulosic bio-ethanol. *Renewable and Sustainable Energy Reviews,* 26**,** 307-321.

CHUANG, Y.-S., LAY, C.-H., SEN, B., CHEN, C.-C., K, G., WU, J.-H., LIN, C.-S. & LIN, C.-Y. 2011. Biohydrogen and biomethane from water hyacinth (Eichhornia crassipes) fermentation: Effects of substrate concentration and incubation temperature. *International Journal of Hydrogen Energy,* 36**,** 14195-14203.

CISNEROS-PÉREZ, C., ETCHEBEHERE, C., CELIS, L. B., CARRILLO-REYES, J., ALATRISTE-MONDRAGÓN, F. & RAZO-FLORES, E. 2017. Effect of inoculum pretreatment on the microbial community structure and its performance during dark fermentation using anaerobic fluidized-bed reactors. *International Journal of Hydrogen Energy,* 42**,** 9589-9599.

CLESCERI, L. S., GREENBERG, A. E. & EATON, A. D. 1998. *Standard Methods for the Examination of Water and Wastewater, 20th Edition,* APHA American Public Health Association.

CLION, V., DUMAS, C., COLLIN, S. & ERNST, B. 2015. Key factors for biohydrogen production by dark fermentation. *The Canadian Journal of Chemical Engineering,* 93**,** 309-316.

COETZEE, J. A. & HILL, M. P. 2011. The role of eutrophication in the biological control of water hyacinth, Eichhornia crassipes, in South Africa. *BioControl,* 57**,** 247-261.

CORNELI, E., ADESSI, A., OLGUÍN, E., RAGAGLINI, G., GARCÍA-LÓPEZ, D. & DE PHILIPPIS, R. 2017. Biotransformation of water lettuce (Pistia stratiotes) to biohydrogen by Rhodopseudomonas palustris. *Journal of applied microbiology,* 123**,** 1438-1446.

CUI, W. & CHENG, J. J. 2015. Growing duckweed for biofuel production: a review. *Plant Biology,* 17**,** 16-23.

DASGUPTA, C. N., JOSE GILBERT, J., LINDBLAD, P., HEIDORN, T., BORGVANG, S. A., SKJANES, K. & DAS, D. 2010. Recent trends on the development of photobiological processes and photobioreactors for the improvement of hydrogen production. *International Journal of Hydrogen Energy,* 35**,** 10218-10238.

DE AMORIM, E. L. C., SADER, L. T. & SILVA, E. L. 2012. Effect of substrate concentration on dark fermentation hydrogen production using an anaerobic fluidized bed reactor. *Applied biochemistry and biotechnology,* 166**,** 1248-1263.

DE SÁ, L. R. V., CAMMAROTA, M. C., DE OLIVEIRA, T. C., OLIVEIRA, E. M. M., MATOS, A. & FERREIRA-LEITÃO, V. S. 2013. Pentoses, hexoses and glycerin as substrates for biohydrogen production: An approach for Brazilian biofuel integration. *International Journal of Hydrogen Energy,* 38**,** 2986-2997.

DE SÁ, L. R. V., DE OLIVEIRA, T. C., DOS SANTOS, T. F., MATOS, A., CAMMAROTA, M. C., OLIVEIRA, E. M. M. & FERREIRA-LEITÃO, V. S. 2011. Hydrogenase activity monitoring in the

fermentative hydrogen production using heat pretreated sludge: A useful approach to evaluate bacterial communities performance. *International Journal of Hydrogen Energy,* 36**,** 7543-7549.

DĘBOWSKI, M., KORZENIEWSKA, E., FILIPKOWSKA, Z., ZIELIŃSKI, M. & KWIATKOWSKI, R. 2014. Possibility of hydrogen production during cheese whey fermentation process by different strains of psychrophilic bacteria. *International journal of hydrogen energy,* 39**,** 1972-1978.

DELBECQ, F., WANG, Y., MURALIDHARA, A., EL OUARDI, K., MARLAIR, G. & LEN, C. 2018. Hydrolysis of Hemicellulose and Derivatives—A Review of Recent Advances in the Production of Furfural. *Frontiers in Chemistry,* 6**,** 146.

DEMIRBAS, A. 2016. Comparison of thermochemical conversion processes of biomass to hydrogen-rich gas mixtures. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects,* 38**,** 2971-2976.

DESSÌ, P., PORCA, E., FRUNZO, L., LAKANIEMI, A. M., COLLINS, G., ESPOSITO, G. & LENS, P. N. L. 2018. Inoculum pretreatment differentially affects the active microbial community performing mesophilic and thermophilic dark fermentation of xylose. *International Journal of Hydrogen Energy,* 43**,** 9233-9245.

DHANASEKAR, R. & JONESH, S. 2018. Identification of a novel hydrogen producing bacteria from sugarcane bagasse waste. *Biocatalysis and Agricultural Biotechnology,* 15**,** 277-282.

DIMITRIOU, P., TSUJIMURA, T. & SUZUKI, Y. 2018. Hydrogen-diesel dual-fuel engine optimization for CHP systems. *Energy,* 160**,** 740-752.

DINCER, I. & ACAR, C. 2015. Review and evaluation of hydrogen production methods for better sustainability. *International Journal of Hydrogen Energy,* 40**,** 11094-11111.

DINCER, I. & JOSHI, A. S. 2013. Hydrogen Production Methods. In: Solar Based Hydrogen Production Systems. *SpringerBriefs in Energy*. *Springer*. New York, NY

DIVYABHARATHI, R., ANGEESWARAN, R., JAGADEESHKUMAR, K. & PUGALENDHI, S. 2017. Characterization and Batch Anaerobic Digestion Study of Banana Wastes. *International Journal of Current Microbiology and Applied Sciences,* 6**,** 2307-2315.

DUBLIN, July 17, 2018. Research and Markets. Petroleum Industry in South Africa 2018: State of the Industry, Influencing Factors & Competition. https://www.prnewswire.com/news-releases/petroleum-industry-in-south-africa-2018-state-of-the-industry-influencing-factors--competition-300682138.html.

DUTTA, S. 2014. A review on production, storage of hydrogen and its utilization as an energy resource. *Journal of Industrial and Engineering Chemistry,* 20**,** 1148-1156.

EL-EMAM, R. S., OZCAN, H. & DINCER, I. 2015. Comparative cost evaluation of nuclear hydrogen production methods with the Hydrogen Economy Evaluation Program (HEEP). *International journal of hydrogen energy,* 40**,** 11168-11177.

ELBESHBISHY, E., DHAR, B. R., NAKHLA, G. & LEE, H.-S. 2017. A critical review on inhibition of dark biohydrogen fermentation. *Renewable and Sustainable Energy Reviews,* 79**,** 656-668.

ELLISTON, A., WILSON, D. R., WELLNER, N., COLLINS, S. R. A., ROBERTS, I. N. & WALDRON, K. W. 2015. Effect of steam explosion on waste copier paper alone and in a mixed lignocellulosic substrate on saccharification and fermentation. *Bioresource Technology,* 187**,** 136-143.

ELSHARNOUBY, O., HAFEZ, H., NAKHLA, G. & EL NAGGAR, M. H. 2013. A critical literature review on biohydrogen production by pure cultures. *International Journal of Hydrogen Energy,* 38**,** 4945-4966.

ENNOURI, H., MILADI, B., DIAZ, S. Z., GÜELFO, L. A. F., SOLERA, R., HAMDI, M. & BOUALLAGUI, H. 2016. Effect of thermal pretreatment on the biogas production and microbial communities balance during anaerobic digestion of urban and industrial waste activated sludge. *Bioresource Technology,* 214**,** 184-191.

EROGLU, E. & MELIS, A. 2016. Microalgal hydrogen production research. *International Journal of Hydrogen Energy,* 41**,** 12772-12798.

FADAIRO, A. & FAGBENLE, R. Biogas production from water Hyacinth blends. 2014. International Conference on Heat Transfer, Fluid Mechanics and Thermodynamics.

FATEHIZADEH, A., AMIN, M. M., BINA, B., ZARE, M. R., GHASEMIAN, M. & TAHERI, E. 2018. Biohydrogen Production as Clean Fuel from physically Pretreated Mixed Culture. *Advanced Biomedical Research,* 7**,** 80.

FLORIO, C., DOMENICO PIROZZIB, ANGELO AUSIELLOB, LUCA MICOLIB, VINCENZO, PASQUALEA, GIUSEPPE TOSCANOB, MARIA TURCOB & DUMONTETA, S. 2017. Effect of Inoculum/Substrate Ratio on Dark Fermentation for

Biohydrogen Production from Organic Fraction of Municipal Solid Waste. *Chemical Engineering Transactions,* 57**,** 175-180.

FRASER, G. C. G., HILL, M. P. & MARTIN, J. A. 2016. Economic evaluation of water loss saving due to the biological control of water hyacinth at New Year's Dam, Eastern Cape province, South Africa. *African Journal of Aquatic Science,* 41**,** 227-234.

GANGULY, A., DAS, S., BHATTACHARYA, A. & SINGH, P. 2013. Studies on the production of xylose from water hyacinth. *Advances in Chemical Science,* 2**,** 1-7.

GARCÍA, C. A., MONCADA, J., ARISTIZÁBAL, V. & CARDONA, C. A. 2017. Techno-economic and energetic assessment of hydrogen production through gasification in the Colombian context: Coffee Cut-Stems case. *International Journal of Hydrogen Energy,* 42**,** 5849-5864.

GAUR, R. Z., KHAN, A. A. & SUTHAR, S. 2017. Effect of thermal pre-treatment on co-digestion of duckweed (Lemna gibba) and waste activated sludge on biogas production. *Chemosphere,* 174**,** 754-763.

GE, X., ZHANG, N., PHILLIPS, G. C. & XU, J. 2012. Growing Lemna minor in agricultural wastewater and converting the duckweed biomass to ethanol. *Bioresource Technology,* 124**,** 485-488.

GHASEMZADEH, K., SADATI TILEBON, S. M. & BASILE, A. 2018. Chapter 9 - Reforming and Partial Oxidation Reactions of Methanol for Hydrogen Production. *In:* BASILE, A. & DALENA, F. (eds.) *Methanol.* Elsevier.

GHIMIRE, A., FRUNZO, L., PIROZZI, F., TRABLY, E., ESCUDIE, R., LENS, P. N. L. & ESPOSITO, G. 2015a. A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products. *Applied Energy,* 144**,** 73-95.

GHIMIRE, A., FRUNZO, L., PONTONI, L., D'ANTONIO, G., LENS, P. N., ESPOSITO, G. & PIROZZI, F. 2015b. Dark fermentation of complex waste biomass for biohydrogen production by pretreated thermophilic anaerobic digestate. *J Environ Manage,* 152**,** 43-8.

GHIMIRE, A., SPOSITO, F., FRUNZO, L., TRABLY, E., ESCUDIE, R., PIROZZI, F., LENS, P. N. & ESPOSITO, G. 2016. Effects of operational parameters on dark fermentative hydrogen production from biodegradable complex waste biomass. *Waste Manag,* 50**,** 55-64.

GHOSH, S., CHOWDHURY, R. & BHATTACHARYA, P. 2018. A review on single stage integrated dark-photo fermentative biohydrogen production: Insight into salient strategies and scopes. *International Journal of Hydrogen Energy*, 43**,** 2091-2107.

GHOSH, S., DAIRKEE, U. K., CHOWDHURY, R. & BHATTACHARYA, P. 2017. Hydrogen from food processing wastes via photofermentation using Purple Non-sulfur Bacteria (PNSB) – A review. *Energy Conversion and Management*, 141**,** 299-314.

GINKEL, S. V., SUNG, S. & LAY, J.-J. 2001. Biohydrogen production as a function of pH and substrate concentration. *Environmental science & technology*, 35**,** 4726-4730.

GIORDANO, A., SARLI, V., LAVAGNOLO, M. C. & SPAGNI, A. 2014. Evaluation of aeration pretreatment to prepare an inoculum for the two-stage hydrogen and methane production process. *Bioresource Technology*, 166**,** 211-218.

GONZALES, R. R., SIVAGURUNATHAN, P. & KIM, S.-H. 2016. Effect of severity on dilute acid pretreatment of lignocellulosic biomass and the following hydrogen fermentation. *International Journal of Hydrogen Energy*, 41**,** 21678-21684.

GONZALEZ-MARTINEZ, A., RODRIGUEZ-SANCHEZ, A., RODELAS, B., ABBAS, B. A., MARTINEZ-TOLEDO, M. V., VAN LOOSDRECHT, M., OSORIO, F. & GONZALEZ-LOPEZ, J. 2015. 454-Pyrosequencing analysis of bacterial communities from autotrophic nitrogen removal bioreactors utilizing universal primers: effect of annealing temperature. *BioMed research international*, 2015.

GORHAM, J. B., WILLIAMS, B. A., GIDLEY, M. J. & MIKKELSEN, D. 2016. Visualization of microbe-dietary remnant interactions in digesta from pigs, by fluorescence in situ hybridization and staining methods; effects of a dietary arabinoxylan-rich wheat fraction. *Food Hydrocolloids*, 52**,** 952-962.

GU, Y., CHEN, X., LIU, Z., ZHOU, X. & ZHANG, Y. 2014. Effect of inoculum sources on the anaerobic digestion of rice straw. *Bioresource Technology*, 158**,** 149-155.

GUO, X. M., TRABLY, E., LATRILLE, E., CARRÈRE, H. & STEYER, J.-P. 2010. Hydrogen production from agricultural waste by dark fermentation: A review. *International Journal of Hydrogen Energy*, 35**,** 10660-10673.

GÜRTEKIN, E. 2014. Biological Hydrogen Production Methods.

GUSAIN, R. & SUTHAR, S. 2017. Potential of aquatic weeds (Lemna gibba , Lemna minor , Pistia stratiotes and Eichhornia sp.) in biofuel production. *Process Safety and Environmental Protection,* 109**,** 233-241.

HALDAR, D., SEN, D. & GAYEN, K. 2016. A review on the production of fermentable sugars from lignocellulosic biomass through conventional and enzymatic route—a comparison. *International Journal of Green Energy,* 13**,** 1232-1253.

HALLENBECK, P. C. & BENEMANN, J. R. 2002. Biological hydrogen production; fundamentals and limiting processes. *International Journal of Hydrogen Energy,* 27**,** 1185-1193.

HANSEN, C. L. & CHEONG, D. Y. 2013. Agricultural Waste Management in Food Processing. 619-666.

HANSEN, M. & PERNER, M. 2016. Hydrogenase Gene Distribution and H2 Consumption Ability within the Thiomicrospira Lineage. *Front Microbiol,* 7**,** 99.

HARON, R., MAT, R., TUAN ABDULLAH, T. A. & RAHMAN, R. A. 2018. Overview on utilization of biodiesel by-product for biohydrogen production. *Journal of Cleaner Production,* 172**,** 314-324.

HARUN, I., JAHIM, J. M., ANUAR, N. & HASSAN, O. 2012. Hydrogen production performance by Enterobacter cloacae KBH3 isolated from termite guts. *International Journal of Hydrogen Energy,* 37**,** 15052-15061.

HARUN, M. Y., DAYANG RADIAH, A. B., ZAINAL ABIDIN, Z. & YUNUS, R. 2011. Effect of physical pretreatment on dilute acid hydrolysis of water hyacinth (Eichhornia crassipes). *Bioresource Technology,* 102**,** 5193-5199.

HILL, M. P. & COETZEE, J. 2017. The biological control of aquatic weeds in South Africa: Current status and future challenges. *Bothalia - African Biodiversity & Conservation,* 47**,** 1-12.

HU, B.-B. & ZHU, M.-J. 2017. Direct hydrogen production from dilute-acid pretreated sugarcane bagasse hydrolysate using the newly isolated Thermoanaerobacterium thermosaccharolyticum MJ1. *Microbial Cell Factories,* 16.

HU, Q., SU, X., TAN, L., LIU, X., WU, A., SU, D., TIAN, K. & XIONG, X. 2013. Effects of a steam explosion pretreatment on sugar production by enzymatic hydrolysis and structural properties of reed straw. *Biosci Biotechnol Biochem,* 77**,** 2181-7.

HUANG, W., WANG, E., CHANG, J., WANG, P., YIN, Q., LIU, C., ZHU, Q. & LU, F. 2017. Effect of Physicochemical Pretreatments and Enzymatic Hydrolysis on Corn Straw Degradation and Reducing Sugar Yield. *BioResources,* 12**,** 7002-7015.

HUNG, C., CHENG, C., CHENG, L., LIANG, C. & LIN, C. 2008. Application of Clostridium-specific PCR primers on the analysis of dark fermentation hydrogen-producing bacterial community. *International Journal of Hydrogen Energy,* 33**,** 1586-1592.

HWANG, J.-H., CHOI, J.-A., ABOU-SHANAB, R. A. I., BHATNAGAR, A., MIN, B., SONG, H., KUMAR, E., CHOI, J., LEE, E. S. & KIM, Y. J. 2009a. Effect of pH and sulfate concentration on hydrogen production using anaerobic mixed microflora. *International Journal of Hydrogen Energy,* 34**,** 9702-9710.

HWANG, J.-H., CHOI, J.-A., ABOU-SHANAB, R. A. I., BHATNAGAR, A., MIN, B., SONG, H., KUMAR, E., CHOI, J., LEE, E. S., KIM, Y. J., UM, S., LEE, D. S. & JEON, B.-H. 2009b. Effect of pH and sulfate concentration on hydrogen production using anaerobic mixed microflora. *International Journal of Hydrogen Energy,* 34**,** 9702-9710.

IM, W.-T., KIM, D.-H., KIM, K.-H. & KIM, M.-S. 2012. Bacterial community analyses by pyrosequencing in dark fermentative H2-producing reactor using organic wastes as a feedstock. *international journal of hydrogen energy,* 37**,** 8330-8337.

JACQUET, N., MANIET, G., VANDERGHEM, C., DELVIGNE, F. & RICHEL, A. 2015. Application of Steam Explosion as Pretreatment on Lignocellulosic Material: A Review. *Industrial & Engineering Chemistry Research,* 54**,** 2593-2598.

JASEENA, K. & SOSAMONY, K. 2016. Practical Aspects of Hydrogen Production by Dark Fermentation–A Review.

JO, J. H., LEE, D. S., PARK, D., CHOE, W.-S. & PARK, J. M. 2008. Optimization of key process variables for enhanced hydrogen production by Enterobacter aerogenes using statistical methods. *Bioresource technology,* 99**,** 2061-2066.

JÖNSSON, L. J. & MARTÍN, C. 2016. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. *Bioresource Technology,* 199**,** 103-112.

JUNG, K.-W., KIM, D.-H. & SHIN, H.-S. 2011. Fermentative hydrogen production from Laminaria japonica and optimization of thermal pretreatment conditions. *Bioresource Technology,* 102**,** 2745-2750.

KAIWEN, L., BIN, Y. & TAO, Z. 2018. Economic analysis of hydrogen production from steam reforming process: A literature review. *Energy Sources, Part B: Economics, Planning, and Policy,* 13**,** 109-115.

KALAMARAS, C. M. & EFSTATHIOU, A. M. Hydrogen production technologies: current state and future developments. Conference papers in science, 2013a. Hindawi.

KALAMARAS, C. M. & EFSTATHIOU, A. M. 2013b. Hydrogen Production Technologies: Current State and Future Developments. *Conference Papers in Energy,* 2013**,** 9.

KAUR, M., KUMAR, M., SACHDEVA, S. & PURI, S. K. 2018. Aquatic weeds as the next generation feedstock for sustainable bioenergy production. *Bioresource Technology,* 251**,** 390-402.

KEIPI, T., TOLVANEN, H. & KONTTINEN, J. 2018. Economic analysis of hydrogen production by methane thermal decomposition: Comparison to competing technologies. *Energy Conversion and Management,* 159**,** 264-273.

KHAMIS, I. & MALSHE, U. 2010. HEEP: a new tool for the economic evaluation of hydrogen economy. *International journal of hydrogen energy,* 35**,** 8398-8406.

KHAN, M. A., NGO, H. H., GUO, W., LIU, Y., ZHANG, X., GUO, J., CHANG, S. W., NGUYEN, D. D. & WANG, J. 2018. Biohydrogen production from anaerobic digestion and its potential as renewable energy. *Renewable Energy,* 129**,** 754-768.

KHANNA, N. & DAS, D. 2013. Biohydrogen production by dark fermentation. *Wiley Interdisciplinary Reviews: Energy and Environment,* 2**,** 401-421.

KIM, D. 2018. Physico-Chemical Conversion of Lignocellulose: Inhibitor Effects and Detoxification Strategies: A Mini Review. *Molecules,* 23.

KIM, Y., KREKE, T., KO, J. K. & LADISCH, M. R. 2015a. Hydrolysis-determining substrate characteristics in liquid hot water pretreated hardwood. *Biotechnol Bioeng,* 112**,** 677-87.

KIM, Y., KREKE, T., KO, J. K. & LADISCH, M. R. 2015b. Hydrolysis-determining substrate characteristics in liquid hot water pretreated hardwood. *Biotechnology and bioengineering,* 112**,** 677-687.

KIRLI, B. & KARAPINAR, I. 2018. The effect of HRT on biohydrogen production from acid hydrolyzed waste wheat in a continuously operated packed bed reactor. *International Journal of Hydrogen Energy*.

KONGJAN, P. & ANGELIDAKI, I. 2010. Extreme thermophilic biohydrogen production from wheat straw hydrolysate using mixed culture fermentation: Effect of reactor configuration. *Bioresource Technology,* 101**,** 7789-7796.

KOTHARI, R., KUMAR, V., PATHAK, V. V., AHMAD, S., AOYI, O. & TYAGI, V. 2017. A critical review on factors influencing fermentative hydrogen production. *Frontiers in bioscience (Landmark edition),* 22**,** 1195-1220.

KRISTIANI, A., ABIMANYU, H., SETIAWAN, A. H., SUDIYARMANTO & AULIA, F. 2013. Effect of Pretreatment Process by Using Diluted Acid to Characteristic of oil Palm's Frond. *Energy Procedia,* 32**,** 183-189.

KUMAR, G., BAKONYI, P., PERIYASAMY, S., KIM, S. H., NEMESTÓTHY, N. & BÉLAFI-BAKÓ, K. 2015. Lignocellulose biohydrogen: Practical challenges and recent progress. *Renewable and Sustainable Energy Reviews,* 44**,** 728-737.

KUMAR, G., CHO, S.-K., SIVAGURUNATHAN, P., ANBURAJAN, P., MAHAPATRA, D. M., PARK, J.-H. & PUGAZHENDHI, A. 2018a. Insights into evolutionary trends in molecular biology tools in microbial screening for biohydrogen production through dark fermentation. *International Journal of Hydrogen Energy,* 43**,** 19885-19901.

KUMAR, G., PARK, J.-H., KIM, M.-S., KIM, D.-H. & KIM, S.-H. 2014. Hydrogen fermentation of different galactose–glucose compositions during various hydraulic retention times (HRTs). *International Journal of Hydrogen Energy,* 39**,** 20625-20631.

KUMAR, G., SHOBANA, S., NAGARAJAN, D., LEE, D.-J., LEE, K.-S., LIN, C.-Y., CHEN, C.-Y. & CHANG, J.-S. 2018b. Biomass based hydrogen production by dark fermentation—recent trends and opportunities for greener processes. *Current Opinion in Biotechnology,* 50**,** 136-145.

KUMAR, G., SIVAGURUNATHAN, P., SEN, B., MUDHOO, A., DAVILA-VAZQUEZ, G., WANG, G. & KIM, S.-H. 2017. Research and development perspectives of lignocellulose-based biohydrogen production. *International Biodeterioration & Biodegradation,* 119**,** 225-238.

KUMARI, S. & DAS, D. 2017. Improvement of biohydrogen production using acidogenic culture. *International Journal of Hydrogen Energy,* 42**,** 4083-4094.

LALMAN, J. A., CHAGANTI, S. R., MOON, C. & KIM, D. H. 2013. Elucidating acetogenic H2 consumption in dark fermentation using flux balance analysis. *Bioresour Technol,* 146**,** 775-8.

LAOTHANACHAREON, T., KANCHANASUTA, S., MHUANTHONG, W., PHALAKORNKULE, C., PISUTPAISAL, N. & CHAMPREDA, V. 2014. Analysis of microbial community adaptation in mesophilic hydrogen fermentation from food waste by tagged 16S rRNA gene pyrosequencing. *J Environ Manage,* 144**,** 143-51.

LAP, L. 1998. Chemical Analysis and Testing Laboratory Analytical Procedures.".

LAXMAN PACHAPUR, V., JYOTI SARMA, S., KAUR BRAR, S., LE BIHAN, Y., RICARDO SOCCOL, C., BUELNA, G. & VERMA, M. 2015. Co-culture strategies for increased biohydrogen production. *International Journal of Energy Research,* 39**,** 1479-1504.

LAY, C. H., SEN, B., CHEN, C. C., WU, J. H., LEE, S. C. & LIN, C. Y. 2013. Co-fermentation of water hyacinth and beverage wastewater in powder and pellet form for hydrogen production. *Bioresour Technol,* 135**,** 610-5.

LEE, H. S. & RITTMANN, B. E. 2009. Evaluation of metabolism using stoichiometry in fermentative biohydrogen. *Biotechnology and bioengineering,* 102**,** 749-758.

LEE, M.-J., SONG, J.-H. & HWANG, S.-J. 2009. Effects of acid pre-treatment on bio-hydrogen production and microbial communities during dark fermentation. *Bioresource Technology,* 100**,** 1491-1493.

LI, D., JIAO, C., HE, W., YAN, Z., YUAN, Y., LI, Z., GUO, Y. & LIU, X. 2016. Comparison of micro-aerobic and anaerobic fermentative hydrogen production from corn straw. *International Journal of Hydrogen Energy,* 41**,** 5456-5464.

LI, J. & SUN, C. 2018. Towards a low carbon economy by removing fossil fuel subsidies? *China Economic Review,* 50**,** 17-33.

LI, R. Y., ZHANG, T. & FANG, H. H. 2011. Application of molecular techniques on heterotrophic hydrogen production research. *Bioresour Technol,* 102**,** 8445-56.

LIN, R., CHENG, J., SONG, W., DING, L., XIE, B., ZHOU, J. & CEN, K. 2015. Characterisation of water hyacinth with microwave-heated alkali pretreatment for enhanced enzymatic digestibility and hydrogen/methane fermentation. *Bioresour Technol,* 182**,** 1-7.

LIU, H. & WANG, G. 2012. Hydrogen production of a salt tolerant strain Bacillus sp. B2 from marine intertidal sludge. *World J Microbiol Biotechnol,* 28**,** 31-7.

LJUNGGREN, M., WALLBERG, O. & ZACCHI, G. 2011. Techno-economic comparison of a biological hydrogen process and a 2nd generation ethanol process using barley straw as feedstock. *Bioresource technology,* 102**,** 9524-9531.

LJUNGGREN, M. & ZACCHI, G. 2010. Techno-economic analysis of a two-step biological process producing hydrogen and methane. *Bioresource technology,* 101**,** 7780-7788.

LORENZ, T. C. 2012. Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *Journal of visualized experiments: JoVE*.

LU, C., ZHANG, Z., ZHOU, X., HU, J., GE, X., XIA, C., ZHAO, J., WANG, Y., JING, Y., LI, Y. & ZHANG, Q. 2018. Effect of substrate concentration on hydrogen production by photo-fermentation in the pilot-scale baffled bioreactor. *Bioresource Technology,* 247**,** 1173-1176.

ŁUKAJTIS, R., HOŁOWACZ, I., KUCHARSKA, K., GLINKA, M., RYBARCZYK, P., PRZYJAZNY, A. & KAMIŃSKI, M. 2018. Hydrogen production from biomass using dark fermentation. *Renewable and Sustainable Energy Reviews,* 91**,** 665-694.

MALIK, A. 2007. Environmental challenge vis a vis opportunity: the case of water hyacinth. *Environ Int,* 33**,** 122-38.

MAMIMIN, C., PRASERTSAN, P., KONGJAN, P. & O-THONG, S. 2017. Effects of volatile fatty acids in biohydrogen effluent on biohythane production from palm oil mill effluent under thermophilic condition. *Electronic Journal of Biotechnology,* 29**,** 78-85.

MATHERI, A. N., NDIWENI, S. N., BELAID, M., MUZENDA, E. & HUBERT, R. 2017. Optimising biogas production from anaerobic co-digestion of chicken manure and organic fraction of municipal solid waste. *Renewable and Sustainable Energy Reviews,* 80**,** 756-764.

MATHEW, A. K., BHUI, I., BANERJEE, S. N., GOSWAMI, R., CHAKRABORTY, A. K., SHOME, A., BALACHANDRAN, S. & CHAUDHURY, S. 2015. Biogas production from locally available aquatic weeds of Santiniketan through anaerobic digestion. *Clean Technologies and Environmental Policy,* 17**,** 1681-1688.

MATHEW, A. K., PARAMESHWARAN, B., SUKUMARAN, R. K. & PANDEY, A. 2016. An evaluation of dilute acid and ammonia fiber explosion pretreatment for cellulosic ethanol production. *Bioresour Technol,* 199**,** 13-20.

MEI, N., POSTEC, A., MONNIN, C., PELLETIER, B., PAYRI, C. E., MÉNEZ, B., FROUIN, E., OLLIVIER, B., ERAUSO, G. & QUÉMÉNEUR, M. 2016. Metagenomic and PCR-based diversity surveys of [FeFe]-hydrogenases combined with isolation of alkaliphilic hydrogen-producing bacteria from the serpentinite-hosted Prony Hydrothermal Field, New Caledonia. *Frontiers in microbiology,* 7**,** 1301.

MIRANDA, A. F., BISWAS, B., RAMKUMAR, N., SINGH, R., KUMAR, J., JAMES, A., RODDICK, F., LAL, B., SUBUDHI, S. & BHASKAR, T. 2016a. Aquatic plant Azolla as the universal feedstock for biofuel production. *Biotechnology for biofuels,* 9**,** 221.

MIRANDA, A. F., BISWAS, B., RAMKUMAR, N., SINGH, R., KUMAR, J., JAMES, A., RODDICK, F., LAL, B., SUBUDHI, S., BHASKAR, T. & MOURADOV, A. 2016b. Aquatic plant Azolla as the universal feedstock for biofuel production. *Biotechnology for Biofuels,* 9**,** 221.

MISHRA, P. & DAS, D. 2014. Biohydrogen production from Enterobacter cloacae IIT-BT 08 using distillery effluent. *International Journal of Hydrogen Energy,* 39**,** 7496-7507.

MISHRA, P., ROY, S. & DAS, D. 2015. Comparative evaluation of the hydrogen production by mixed consortium, synthetic co-culture and pure culture using distillery effluent. *Bioresour Technol,* 198**,** 593-602.

MISHRA, P., THAKUR, S., SINGH, L., KRISHNAN, S., SAKINAH, M. & AB WAHID, Z. 2017a. Fermentative hydrogen production from indigenous mesophilic strain Bacillus anthracis PUNAJAN 1 newly isolated from palm oil mill effluent. *international journal of hydrogen energy,* 42**,** 16054-16063.

MISHRA, P., THAKUR, S., SINGH, L., KRISHNAN, S., SAKINAH, M. & WAHID, Z. A. 2017b. Fermentative hydrogen production from indigenous mesophilic strain Bacillus anthracis PUNAJAN 1 newly isolated from palm oil mill effluent. *International Journal of Hydrogen Energy,* 42**,** 16054-16063.

MITSUSHIMA, S. & HACKER, V. 2018. Chapter 11 - Role of Hydrogen Energy Carriers. *In:* HACKER, V. & MITSUSHIMA, S. (eds.) *Fuel Cells and Hydrogen.* Elsevier.

MOE, S. T., JANGA, K. K., HERTZBERG, T., HÄGG, M.-B., ØYAAS, K. & DYRSET, N. 2012. Saccharification of Lignocellulosic Biomass for Biofuel and Biorefinery Applications – A Renaissance for the Concentrated Acid Hydrolysis? *Energy Procedia,* 20**,** 50-58.

MOHAMMADI, P., IBRAHIM, S., ANNUAR, M. S. M., GHAFARI, S., VIKINESWARY, S. & ZINATIZADEH, A. A. 2012. Influences of environmental and operational factors on dark fermentative hydrogen production: a review. *CLEAN–Soil, Air, Water,* 40**,** 1297-1305.

MOHANRAJ, S., ANBALAGAN, K., RAJAGURU, P. & PUGALENTHI, V. 2016. Effects of phytogenic copper nanoparticles on fermentative hydrogen production by Enterobacter cloacae and Clostridium acetobutylicum. *International Journal of Hydrogen Energy,* 41**,** 10639-10645.

MØLLER, K. T., JENSEN, T. R., AKIBA, E. & LI, H.-W. 2017. Hydrogen - A sustainable energy carrier. *Progress in Natural Science: Materials International,* 27**,** 34-40.

MONTECCHIO, D., YUAN, Y. & MALPEI, F. 2018. Hydrogen production dynamic during cheese whey Dark Fermentation: New insights from modelization. *International Journal of Hydrogen Energy,* 43**,** 17588-17601.

MOODLEY, P. & GUEGUIM KANA, E. B. 2017. Development of a steam or microwave-assisted sequential salt-alkali pretreatment for lignocellulosic waste: Effect on delignification and enzymatic hydrolysis. *Energy Conversion and Management,* 148**,** 801-808.

MOTA, C., JASON SO, M. & DE LOS REYES, F. 2012. *Identification of Nitrite-Reducing Bacteria Using Sequential mRNA Fluorescence In Situ Hybridization and Fluorescence-Assisted Cell Sorting*.

MU, Y., YANG, H.-Y., WANG, Y.-Z., HE, C.-S., ZHAO, Q.-B., WANG, Y. & YU, H.-Q. 2014. The maximum specific hydrogen-producing activity of anaerobic mixed cultures: definition and determination. *Scientific Reports,* 4**,** 5239.

MYAT, L. & RYU, G. 2016. Pretreatments and factors affecting saccharification and fermentation for lignocellulosic ethanol production. *Cellulose Chem. Technol,* 50**,** 177-188.

NAMADI M, D.M, B., DANGMWAN D.S & A.F, A. 2013. Evaluation of Sugar

Content and Bioethanol Potentials of Some Freshwater Biomass. *International Journal of Renewable and Sustainable Energy,* Vol. 2**,**, pp. 201-204.

NAMADI, M. M. 2013. Evaluation of Sugar Content and Bioethanol Potentials of Some Freshwater Biomass. *International Journal of Renewable and Sustainable Energy,* 2.

NARBEL, P. A. & HANSEN, J. P. 2014. Estimating the cost of future global energy supply. *Renewable and Sustainable Energy Reviews,* 34**,** 91-97.

NASR, M., TAWFIK, A., OOKAWARA, S. & SUZUKI, M. 2013. Environmental and economic aspects of hydrogen and methane production from starch wastewater industry. *Journal of Water and Environment Technology,* 11**,** 463-475.

NIKOLAIDIS, P. & POULLIKKAS, A. 2017. A comparative overview of hydrogen production processes. *Renewable and Sustainable Energy Reviews,* 67**,** 597-611.

NING, Y.-Y., JIN, D.-W., SHENG, G.-P., HARADA, H. & SHI, X.-Y. 2012. Evaluation of the stability of hydrogen production and microbial diversity by anaerobic sludge with chloroform treatment. *Renewable Energy,* 38**,** 253-257.

NISSILÄ, M. E., LAY, C.-H. & PUHAKKA, J. A. 2014. Dark fermentative hydrogen production from lignocellulosic hydrolyzates – A review. *Biomass and Bioenergy,* 67**,** 145-159.

NITIPAN, S., MAMIMIN, C., INTRASUNGKHA, N., BIRKELAND, N. K. & O-THONG, S. 2014. Microbial community analysis of thermophilic mixed culture sludge for biohydrogen production from palm oil mill effluent. *International Journal of Hydrogen Energy,* 39**,** 19285-19293.

NIU, Q., HOJO, T., QIAO, W., QIANG, H. & LI, Y.-Y. 2014. Characterization of methanogenesis, acidogenesis and hydrolysis in thermophilic methane fermentation of chicken manure. *Chemical Engineering Journal,* 244**,** 587-596.

O'SULLIVAN, C., ROUNSEFELL, B., GRINHAM, A., CLARKE, W. & UDY, J. 2010. Anaerobic digestion of harvested aquatic weeds: water hyacinth (Eichhornia crassipes), cabomba (Cabomba Caroliniana) and salvinia (Salvinia molesta). *Ecological Engineering,* 36**,** 1459-1468.

OFOEFULE, A. U., OKORO, U. C. & ONUKWULI, O. D. 2015. Effect of hydrolysis reaction time on the reducing sugar yield of Tacca (Tacca involucrata) and Tigernut (Cyperus esculentus) starch for Bioethanol production. *Der Pharma Chemica,* 7**,** 324-329.

ORTIGUEIRA, J., PINTO, T., GOUVEIA, L. & MOURA, P. 2015. Production and storage of biohydrogen during sequential batch fermentation of Spirogyra hydrolyzate by Clostridium butyricum. *Energy,* 88**,** 528-536.

PACHAPUR, V. L., SARMA, S. J., BRAR, S. K., LE BIHAN, Y., BUELNA, G. & VERMA, M. 2015. Biological hydrogen production using co-culture versus mono-culture system. *Environmental Technology Reviews,* 4**,** 55-70.

PALOMO-BRIONES, R., RAZO-FLORES, E., BERNET, N. & TRABLY, E. 2017. Dark-fermentative biohydrogen pathways and microbial networks in continuous stirred tank reactors: Novel insights on their control. *Applied Energy,* 198**,** 77-87.

PAN, J., ZHANG, R., ELMASHAD, H., SUN, H. & YING, Y. 2008. Effect of food to microorganism ratio on biohydrogen production from food waste via anaerobic fermentation. *International Journal of Hydrogen Energy,* 33**,** 6968-6975.

PANDU, K. & JOSEPH, S. 2012. Comparisons and limitations of biohydrogen

PRODUCTION PROCESSES: A REVIEW. *International Journal of Advances in Engineering & Technology,* 2**,** 342-356.

PANETTA, F. & GOODEN, B. 2017. Managing for biodiversity: impact and action thresholds for invasive plants in natural ecosystems. *NeoBiota,* 34**,** 53-66.

PANTAWONG, R., CHUANCHAI, A., THIPBUNRAT, P., UNPAPROM, Y. & RAMARAJ, R. 2015. Experimental investigation of biogas production from water lettuce, Pistia stratiotes L. *Emergent Life Sciences Research,* 1**,** 14-46.

PATEL, S. K. S., KUMAR, P., MEHARIYA, S., PUROHIT, H. J., LEE, J.-K. & KALIA, V. C. 2014. Enhancement in hydrogen production by co-cultures of Bacillus and Enterobacter. *International Journal of Hydrogen Energy,* 39**,** 14663-14668.

PATTRA, S., SANGYOKA, S., BOONMEE, M. & REUNGSANG, A. 2008. Bio-hydrogen production from the fermentation of sugarcane bagasse hydrolysate by Clostridium butyricum. *International Journal of Hydrogen Energy,* 33**,** 5256-5265.

PATTRA, S. & SITTIJUNDA, S. 2015. Optimization of Factors Affecting Acid Hydrolysis of Water Hyacinth Stem (Eichhornia Crassipes) for Bio-Hydrogen Production. *Energy Procedia,* 79**,** 833-837.

PAUDEL, S. R., BANJARA, S. P., CHOI, O. K., PARK, K. Y., KIM, Y. M. & LEE, J. W. 2017. Pretreatment of agricultural biomass for anaerobic digestion: Current state and challenges. *Bioresour Technol,* 245**,** 1194-1205.

PENNISTON, J. & GUEGUIM KANA, E. B. 2018. Impact of medium pH regulation on biohydrogen production in dark fermentation process using suspended and immobilized microbial cells. *Biotechnology & Biotechnological Equipment,* 32**,** 204-212.

PHAM, V. & KIM, J. 2012. Cultivation of unculturable soil bacteria. *TRENDS in Biotechnology*. 30.9. 475-484.

PIELHOP, T., AMGARTEN, J., VON ROHR, P. R. & STUDER, M. H. 2016. Steam explosion pretreatment of softwood: the effect of the explosive decompression on enzymatic digestibility. *Biotechnology for Biofuels,* 9**,** 152.

PITERINA, A. V. & PEMBROKE, J. T. 2013. Use of PCR-DGGE based molecular methods to analyse microbial community diversity and stability during the thermophilic stages of an ATAD wastewater sludge treatment process as an aid to performance monitoring. *ISRN biotechnology,* 2013.

PRAKASH, J., SHARMA, R., PATEL, S. K. S., KIM, I.-W. & KALIA, V. C. 2018. Bio-hydrogen production by co-digestion of domestic wastewater and biodiesel industry effluent. *PLOS ONE,* 13**,** e0199059.

PUGAZHENDHI, A., ANBURAJAN, P., PARK, J.-H., KUMAR, G., SIVAGURUNATHAN, P. & KIM, S.-H. 2017a. Process performance of biohydrogen production using glucose at various HRTs and assessment of microbial dynamics variation via q-PCR. *International Journal of Hydrogen Energy,* 42**,** 27550-27557.

PUGAZHENDHI, A., ANBURAJAN, P., PARK, J.-H., KUMAR, G., SIVAGURUNATHAN, P. & KIM, S.-H. 2017b. Process performance of biohydrogen production using glucose at various HRTs and assessment of microbial dynamics variation via q-PCR. *International Journal of Hydrogen Energy*.

QIAN, C.-X., CHEN, L.-Y., RONG, H. & YUAN, X.-M. 2011. Hydrogen production by mixed culture of several facultative bacteria and anaerobic bacteria. *Progress in Natural Science: Materials International,* 21**,** 506-511.

QIU, C., ZHENG, Y., ZHENG, J., LIU, Y., XIE, C. & SUN, L. 2016. Mesophilic and Thermophilic Biohydrogen Production from Xylose at Various Initial pH and Substrate Concentrations with Microflora Community Analysis. *Energy & Fuels*.

RABEMANOLONTSOA, H. & SAKA, S. 2016. Various pretreatments of lignocellulosics. *Bioresour Technol,* 199**,** 83-91.

RAFIEENIA, R., LAVAGNOLO, M. C. & PIVATO, A. 2018a. Pre-treatment technologies for dark fermentative hydrogen production: Current advances and future directions. *Waste Management,* 71**,** 734-748.

RAFIEENIA, R., PIVATO, A., SCHIEVANO, A. & LAVAGNOLO, M. C. 2018b. Dark fermentation metabolic models to study strategies for hydrogen consumers inhibition. *Bioresource Technology,* 267**,** 445-457.

RAI, P. 2016. Recent Advances in substrate utilization for fermentative hydrogen Production. *Journal of Applied Biology and Biotechnology***,** 059-067.

RAMPRAKASH, B. & MUTHUKUMAR, K. 2015. Comparative study on the performance of various pretreatment and hydrolysis methods for the production of biohydrogen using Enterobacter aerogenes RM 08 from rice mill wastewater. *International journal of Hydrogen energy,* 40**,** 9106-9112.

REDDY, K. 2016. *Evaluation of biohydrogen production potential of sugarcane bagasse using activated sludge in a dark fermentation process.*

REDDY, K., NASR, M., KUMARI, S., KUMAR, S., GUPTA, S. K., ENITAN, A. M. & BUX, F. 2017. Biohydrogen production from sugarcane bagasse hydrolysate: effects of pH, S/X, Fe2+, and magnetite nanoparticles. *Environ Sci Pollut Res Int,* 24**,** 8790-8804.

REZANIA, S., DIN, M. F. M., TAIB, S. M., SOHAILI, J., CHELLIAPAN, S., KAMYAB, H. & SAHA, B. B. 2017. Review on fermentative biohydrogen production from water hyacinth, wheat straw and rice straw with focus on recent perspectives. *International Journal of Hydrogen Energy,* 42**,** 20955-20969.

REZANIA, S., PONRAJ, M., DIN, M. F. M., SONGIP, A. R., SAIRAN, F. M. & CHELLIAPAN, S. 2015. The diverse applications of water hyacinth with main focus on sustainable energy and production for new era: An overview. *Renewable and Sustainable Energy Reviews,* 41**,** 943-954.

RICKARD, L. N., YANG, Z. J. & SCHULDT, J. P. 2016. Here and now, there and then: How "departure dates" influence climate change engagement. *Global Environmental Change,* 38**,** 97-107.

RISMILLER, S. C., GROVES, M. M., MENG, M., DONG, Y. & LIN, J. 2018. Water assisted liquefaction of lignocellulose biomass by ReaxFF based molecular dynamic simulations. *Fuel,* 215**,** 835-843.

ROSSELLI, R., ROMOLI, O., VITULO, N., VEZZI, A., CAMPANARO, S., DE PASCALE, F., SCHIAVON, R., TIARCA, M., POLETTO, F. & CONCHERI, G. 2016. Direct 16S rRNA-seq from bacterial communities: a PCR-independent approach to simultaneously assess microbial diversity and functional activity potential of each taxon. *Scientific reports,* 6**,** 32165.

SAGovNews. 2018. Petrol prices explained: This is why fuel costs are soaring in South Africa. https://www.thesouthafrican.com/petrol-prices-why-rising-causes.

SALEM, A. H., BRUNSTERMANN, R., MIETZEL, T. & WIDMANN, R. 2018. Effect of pre-treatment and hydraulic retention time on biohydrogen production from organic wastes. *International Journal of Hydrogen Energy,* 43**,** 4856-4865.

SANTOS, D. M. F., SEQUEIRA, C. A. C. & FIGUEIREDO, J. L. 2013. Hydrogen production by alkaline water electrolysis. *Química Nova,* 36**,** 1176-1193.

SARTO, S., HILDAYATI, R. & SYAICHURROZI, I. 2019. Effect of chemical pretreatment using sulfuric acid on biogas production from water hyacinth and kinetics. *Renewable Energy,* 132**,** 335-350.

SATHYAPRAKASAN, P. & KANNAN, G. 2015. Economics of bio-hydrogen production. *International Journal of Environmental Science and Development,* 6**,** 352.

SEN, U., SHAKDWIPEE, M. & BANERJEE, R. 2008. Status of biological hydrogen production.

SETLOW, P. 2006. Spores of Bacillus subtilis: their resistance to and killing by radiation, heat and chemicals. *Journal of Applied Microbiology,* 101**,** 514-525.

SHATERZADEH, M. J. & ATAEI, S. A. 2017. The effects of temperature, initial pH, and glucose concentration on biohydrogen production from Clostridium acetobutylicum. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects,* 39**,** 1118-1123.

SHI, X. S., DONG, J. J., YU, J. H., YIN, H., HU, S. M., HUANG, S. X. & YUAN, X. Z. 2017. Effect of Hydraulic Retention Time on Anaerobic Digestion of Wheat Straw in the Semicontinuous Continuous Stirred-Tank Reactors. *Biomed Res Int,* 2017**,** 2457805.

SHOW, K. Y., LEE, D. J. & CHANG, J. S. 2011. Bioreactor and process design for biohydrogen production. *Bioresour Technol,* 102**,** 8524-33.

SI, B., LI, J., LI, B., ZHU, Z., SHEN, R., ZHANG, Y. & LIU, Z. 2015a. The role of hydraulic retention time on controlling methanogenesis and homoacetogenesis in biohydrogen production using upflow anaerobic sludge blanket (UASB) reactor and packed bed reactor (PBR). *International Journal of Hydrogen Energy,* 40**,** 11414-11421.

SI, B., LI, J., LI, B., ZHU, Z., SHEN, R., ZHANG, Y. & LIU, Z. 2015b. *The role of hydraulic retention time on controlling methanogenesis and homoacetogenesis in biohydrogen production using upflow anaerobic sludge blanket (UASB) reactor and packed bed reactor (PBR).*

SILVA-ILLANES, F., TAPIA-VENEGAS, E., SCHIAPPACASSE, M. C., TRABLY, E. & RUIZ-FILIPPI, G. 2017. Impact of hydraulic retention time (HRT) and pH on dark fermentative hydrogen production from glycerol. *Energy,* 141**,** 358-367.

SILVA, F. M. S., MAHLER, C. F., OLIVEIRA, L. B. & BASSIN, J. P. 2018a. Hydrogen and methane production in a two-stage anaerobic digestion system by co-digestion of food waste, sewage sludge and glycerol. *Waste Management,* 76**,** 339-349.

SILVA, J. S., MENDES, J. S., CORREIA, J. A. C., ROCHA, M. V. P. & MICOLI, L. 2018b. Cashew apple bagasse as new feedstock for the hydrogen production using dark fermentation process. *Journal of Biotechnology,* 286**,** 71-78.

SINGH, A. & BISHNOI, N. R. 2013. Comparative study of various pretreatment techniques for ethanol production from water hyacinth. *Industrial Crops and Products,* 44**,** 283-289.

SINGH, A., SEVDA, S., ABU REESH, I., VANBROEKHOVEN, K., RATHORE, D. & PANT, D. 2015. Biohydrogen Production from Lignocellulosic Biomass: Technology and Sustainability. *Energies,* 8**,** 13062-13080.

SINGH, L. & WAHID, Z. A. 2015. Methods for enhancing bio-hydrogen production from biological process: A review. *Journal of Industrial and Engineering Chemistry,* 21**,** 70-80.

SIVAGURUNATHAN, P., KUMAR, G., BAKONYI, P., KIM, S.-H., KOBAYASHI, T., XU, K. Q., LAKNER, G., TÓTH, G., NEMESTÓTHY, N. & BÉLAFI-BAKÓ, K. 2016a. A critical review on

issues and overcoming strategies for the enhancement of dark fermentative hydrogen production in continuous systems. *international journal of hydrogen energy,* 41**,** 3820-3836.

SIVAGURUNATHAN, P., KUMAR, G., KIM, S.-H., KOBAYASHI, T., XU, K.-Q., GUO, W. & HAO NGO, H. 2016b. Enhancement strategies for hydrogen production from wastewater: a review. *Current Organic Chemistry*, 20**,** 2744-2752.

SIVASANKARI, B. & DAVID RAVINDRAN, A. 2016. A Study on Chemical Analysis of Water Hyacinth (Eichornia crassipes), Water Lettuce (Pistia stratiotes). *International Journal of Innovative Research in Science, Engineering and Technology,* 5**,** 17566-17570.

SLUITER, A., B HAMES , R RUIZ, C SCARLATA, J SLUITER, AND, D. T. & CROCKER, D. 2011. Determination of Structural Carbohydrates and Lignin in Biomass. *National Renewable Energy LaboratorySS*.

SOŁOWSKI, G., SHALABY, M. S., ABDALLAH, H., SHABAN, A. M. & CENIAN, A. 2018. Production of hydrogen from biomass and its separation using membrane technology. *Renewable and Sustainable Energy Reviews,* 82**,** 3152-3167.

SONG, W., CHENG, J., DING, L., LIU, M., ZHOU, J. & CEN, K. 2017. Improving fermentative hydrogen production from water hyacinth with genetically modified bacteria. *Environmental Progress & Sustainable Energy,* 36**,** 1296-1300.

SONG, Z.-X., LI, W.-W., LI, X.-H., DAI, Y., PENG, X.-X., FAN, Y.-T. & HOU, H.-W. 2013. Isolation and characterization of a new hydrogen-producing strain Bacillus sp. FS2011. *International Journal of Hydrogen Energy,* 38**,** 3206-3212.

SOTELO-NAVARRO, P., POGGI-VARALDO, H., TURPIN-MARION, S., VÁZQUEZ-MORILLAS, A., BELTRÁN-VILLAVICENCIO, M. & ESPINOSA-VALDEMAR, R. 2017. Biohydrogen production from used diapers: Evaluation of effect of temperature and substrate conditioning. *Waste Management & Research,* 35**,** 267-275.

SRITRAKUL, N., NITISINPRASERT, S. & KEAWSOMPONG, S. 2018. Evaluation of dilute acid pretreatment for bioethanol fermentation from sugarcane bagasse pith. *Agriculture and Natural Resources*.

STERK, A., SCHIJVEN, J., DE RODA HUSMAN, A. M. & DE NIJS, T. 2016. Effect of climate change on runoff of Campylobacter and Cryptosporidium from land to surface water. *Water Research,* 95**,** 90-102.

SU, H., CHENG, J., ZHOU, J., SONG, W. & CEN, K. 2010. Hydrogen production from water hyacinth through dark- and photo- fermentation. *International Journal of Hydrogen Energy,* 35**,** 8929-8937.

SUBUDHI, S., NAYAK, T., RAM KUMAR, N., VIJAYANANTH, P. & LAL, B. 2013. Impact of regulated pH on proto scale hydrogen production from xylose by an alkaline tolerant novel bacterial strain, Enterobacter cloacae DT-1. *International Journal of Hydrogen Energy,* 38**,** 2728-2737.

SUKASEM, N. & PRAYOONKHAM, S. 2017. Biomethane Recovery from Fresh and Dry Water Hyacinth Anaerobic Co-Digestion with Pig Dung, Elephant Dung and Bat Dung with Different Alkali Pretreatments. *Energy Procedia,* 138**,** 294-300.

SUN, L., HUANG, A., GU, W., MA, Y., ZHU, D. & WANG, G. 2015. Hydrogen production by Enterobacter cloacae isolated from sugar refinery sludge. *International Journal of Hydrogen Energy,* 40**,** 1402-1407.

SUN, S., SUN, S., CAO, X. & SUN, R. 2016. The role of pretreatment in improving the enzymatic hydrolysis of lignocellulosic materials. *Bioresour Technol,* 199**,** 49-58.

SUNYOTO, N. M., ZHU, M., ZHANG, Z. & ZHANG, D. 2017. Effect of Biochar Addition and Initial pH on Hydrogen Production from the First Phase of Two-Phase Anaerobic Digestion of Carbohydrates Food Waste. *Energy Procedia,* 105**,** 379-384.

SUTTHIPATTANASOMBOON, C. & WONGTHANATE, J. 2017. Enhancement of biohydrogen production from starch processing wastewater and further inside its ecosystem disclosed by 16S rDNA sequencing and FISH. *Brazilian Archives of Biology and Technology,* 60.

THANWISED, P., WIROJANAGUD, W. & REUNGSANG, A. 2012. Effect of hydraulic retention time on hydrogen production and chemical oxygen demand removal from tapioca wastewater using anaerobic mixed cultures in anaerobic baffled reactor (ABR). *International Journal of Hydrogen Energy,* 37**,** 15503-15510.

TOLVANEN, K. E. S., KOSKINEN, P. E. P., YLIKOSKI, A. I., OLLIKKA, P. K., HEMMILÄ, I. A., PUHAKKA, J. A. & KARP, M. T. 2008. Quantitative monitoring of a hydrogen-producing

Clostridium butyricum strain from a continuous-flow, mixed culture bioreactor employing real-time PCR. *International Journal of Hydrogen Energy,* 33**,** 542-549.

TUNÇAY, E. G., ERGUDER, T. H., EROĞLU, İ. & GÜNDÜZ, U. 2017. Dark fermentative hydrogen production from sucrose and molasses. *International Journal of Energy Research,* 41**,** 1891-1902.

TUTT, M. 2015. *Factors affecting biochemical composition of lignocellulosic biomass and its effect on selection of pretreatment method and on bioethanol production potential.* Eesti Maaülikool.

USMANI, M. A., KHAN, I., HAQUE, A., BHAT, A. H., MONDAL, D. & GAZAL, U. 2017. 4 - Biomass-based composites from different sources: Properties, characterization, and transforming biomass with ionic liquids. *In:* JAWAID, M., MD TAHIR, P. & SABA, N. (eds.) *Lignocellulosic Fibre and Biomass-Based Composite Materials.* Woodhead Publishing.

VAN WILGEN, B. W., FILL, J. M., GOVENDER, N. & FOXCROFT, L. C. 2017. An assessment of the evolution, costs and effectiveness of alien plant control operations in Kruger National Park, South Africa. *NeoBiota,* 35**,** 35-59.

VARANASI, J. L., KUMARI, S. & DAS, D. 2018. Improvement of energy recovery from water hyacinth by using integrated system. *International Journal of Hydrogen Energy,* 43**,** 1303-1318.

VARRONE, C., HEGGESET, T. M. B., LE, S. B., HAUGEN, T., MARKUSSEN, S., SKIADAS, I. V. & GAVALA, H. N. 2015. Comparison of Different Strategies for Selection/Adaptation of Mixed Microbial Cultures Able to Ferment Crude Glycerol Derived from Second-Generation Biodiesel. *BioMed Research International,* 2015**,** 932934.

VERARDI, A., BLASI, A., MARINO, T., MOLINO, A. & CALABRÒ, V. 2018. Effect of steam-pretreatment combined with hydrogen peroxide on lignocellulosic agricultural wastes for bioethanol production: Analysis of derived sugars and other by-products. *Journal of Energy Chemistry,* 27**,** 535-543.

VERGARA, P., WOJTUSIK, M., REVILLA, E., LADERO, M., GARCIA-OCHOA, F. & VILLAR, J. C. 2018. Wheat straw fractionation by ethanol-water mixture: Optimization of operating conditions and comparison with diluted sulfuric acid pre-treatment. *Bioresour Technol,* 256**,** 178-186.

VI, L. V. T., SALAKKAM, A. & REUNGSANG, A. 2017. Optimization of key factors affecting bio-hydrogen production from sweet potato starch. *Energy Procedia,* 138**,** 973-978.

WALIGÓRSKA, M. 2012. Fermentative Hydrogen Production - Process Design and Bioreactors. *Chemical and Process Engineering.*

WANG, J. & WAN, W. 2009. Factors influencing fermentative hydrogen production: A review. *International Journal of Hydrogen Energy,* 34**,** 799-811.

WANG, J. & YIN, Y. 2017. Principle and application of different pretreatment methods for enriching hydrogen-producing bacteria from mixed cultures. *International Journal of Hydrogen Energy,* 42**,** 4804-4823.

WANG, J. & YIN, Y. 2018. Fermentative hydrogen production using various biomass-based materials as feedstock. *Renewable and Sustainable Energy Reviews,* 92**,** 284-306.

WANG, S., MA, Z., ZHANG, T., BAO, M. & SU, H. 2017. Optimization and modeling of biohydrogen production by mixed bacterial cultures from raw cassava starch. *Frontiers of Chemical Science and Engineering,* 11**,** 100-106.

WANG, X., HOEFEL, D., SAINT, C. P., MONIS, P. T. & JIN, B. 2007. The isolation and microbial community analysis of hydrogen producing bacteria from activated sludge. *J Appl Microbiol,* 103**,** 1415-23.

WANGER, A., CHAVEZ, V., HUANG, R. S. P., WAHED, A., ACTOR, J. K. & DASGUPTA, A. 2017. Chapter 5 - Biochemical Tests and Staining Techniques for Microbial Identification. *In:* WANGER, A., CHAVEZ, V., HUANG, R. S. P., WAHED, A., ACTOR, J. K. & DASGUPTA, A. (eds.) *Microbiology and Molecular Diagnosis in Pathology.* Elsevier.

WON, S., BALDWIN, S., LAU, A. & REZADEHBASHI, M. 2013. Optimal operational conditions for biohydrogen production from sugar refinery wastewater in an ASBR. *international journal of hydrogen energy,* 38**,** 13895-13906.

WONG, Y. M., SHOW, P. L., WU, T. Y., LEONG, H. Y., IBRAHIM, S. & JUAN, J. C. 2018. Production of bio-hydrogen from dairy wastewater using pretreated landfill leachate sludge as an inoculum. *Journal of Bioscience and Bioengineering*.

WONGTHANATE, J. & CHINNACOTPONG, K. 2015. Optimal conditions for biological hydrogen production from food waste. *Environmental Engineering Research,* 20**,** 121-125.

XU, J. & DESHUSSES, M. A. 2015. Fermentation of swine wastewater-derived duckweed for biohydrogen production. *International Journal of Hydrogen Energy,* 40**,** 7028-7036.

XU, J. L., CUI, W. H., CHENG, J. J. & STOMP, A. M. 2011. Production of high-starch duckweed and its conversion to bioethanol. *Biosyst Eng,* 110.

YANG, G. & WANG, J. 2018. Various additives for improving dark fermentative hydrogen production: A review. *Renewable and Sustainable Energy Reviews,* 95**,** 130-146.

YUNOS, N. S. H. M., BAHARUDDIN, A. S., YUNOS, K. F. M., NAIM, M. N. & NISHIDA, H. 2012. Physicochemical property changes of oil palm mesocarp fibers treated with high-pressure steam. *BioResources,* 7**,** 5983-5994.

ZAGRODNIK, R. & ŁANIECKI, M. 2017. The effect of pH on cooperation between dark- and photo-fermentative bacteria in a co-culture process for hydrogen production from starch. *International Journal of Hydrogen Energy,* 42**,** 2878-2888.

ZHANG, Q., ZHANG, Z., WANG, Y., LEE, D.-J., LI, G., ZHOU, X., JIANG, D., XU, B., LU, C., LI, Y. & GE, X. 2018. Sequential dark and photo fermentation hydrogen production from hydrolyzed corn stover: A pilot test using 11 m3 reactor. *Bioresource Technology*.

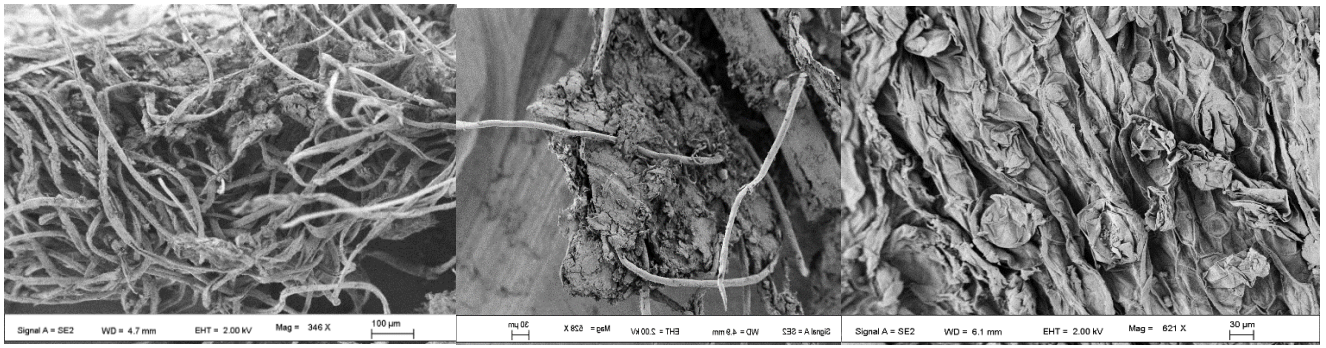
ZHANG, T., BAO, M., WANG, Y., SU, H. & TAN, T. 2014. Genome Sequence of Bacillus cereus Strain A1, an Efficient Starch-Utilizing Producer of Hydrogen. *Genome Announc,* 2.

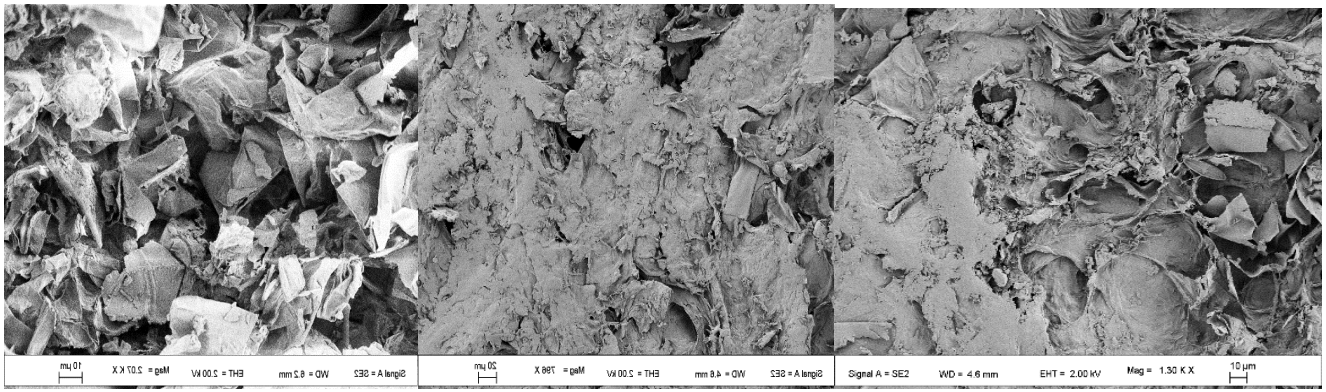
ZHAO, S., LI, G., ZHENG, N., WANG, J. & YU, Z. 2018. Steam explosion enhances digestibility and fermentation of corn stover by facilitating ruminal microbial colonization. *Bioresour Technol,* 253**,** 244-251.

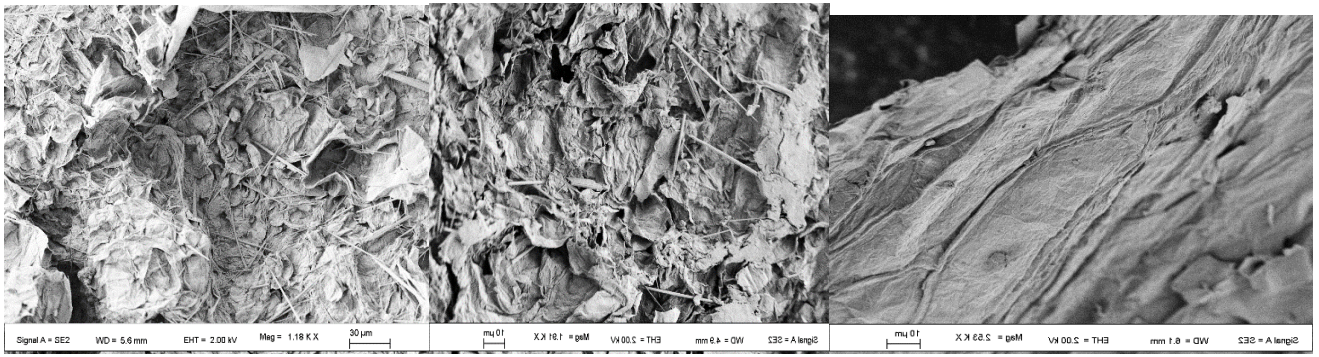
ZHENG, H., ZENG, R. J., O'SULLIVAN, C. & CLARKE, W. P. 2016. Critical analysis of hydrogen production from mixed culture fermentation under thermophilic condition (60 degrees C). *Appl Microbiol Biotechnol,* 100**,** 5165-76.

APPENDICES

APPENDIX ONE (SEM)







APPENDIX TWO: (Chapter IV)

Determination of stoichiometric reactions at various environmental conditions. These steps were employed according to Lee and Rittmann (Amin et al.) Lee H-S, Rittmann BE. Evaluation of Metabolism Using Stoichiometry in Fermentative Biohydrogen. Biotechnol Bioeng 2009; 102:749–758.

Step#1: Calculations of e^-_{ai}, which is the fraction of e^-_{eq} for i product of total e^-eq. For calculations accuracy, the sum of e^-_{ai} should be equals unity.

Culture pH	Acetic acid	Butyric acid	Propionic acid	Lactic acid	H_2	Sum
4.5	0.386	0.306	0.059	0.037	0.212	1.000
5.5	0.378	0.276	0.060	0.030	0.256	1.000
6.5	0.378	0.280	0.065	0.028	0.248	1.000
7.5	0.401	0.295	0.092	0.042	0.170	1.000
8.5	0.384	0.294	0.117	0.046	0.160	1.000

Fermentation time (h)	Acetic acid	Butyric acid	Propionic acid	Lactic acid	H_2	Sum
4	0.380	0.275	0.084	0.051	0.210	1.000
6	0.394	0.294	0.070	0.015	0.227	1.000

8	0.390	0.301	0.052	0.010	0.247	1.000
10	0.392	0.286	0.077	0.006	0.239	1.000
12	0.356	0.282	0.092	0.023	0.247	1.000

Culture temperature (°C)	Acetic acid	Butyric acid	Propionic acid	Lactic acid	H_2	Sum
15	0.356	0.280	0.089	0.058	0.217	1.000
20	0.382	0.297	0.068	0.015	0.238	1.000
25	0.357	0.286	0.068	0.038	0.250	1.000
30	0.361	0.294	0.056	0.035	0.254	1.000
35	0.380	0.297	0.050	0.009	0.264	1.000

S/X	Acetic acid	Butyric acid	Propionic acid	Lactic acid	H_2	Sum
0.5	0.378	0.281	0.031	0.021	0.290	1.000
1.0	0.392	0.305	0.037	0.008	0.257	1.000
1.5	0.369	0.344	0.072	0.040	0.175	1.000
2.0	0.422	0.353	0.088	0.005	0.132	1.000
2.5	0.442	0.350	0.074	0.036	0.099	1.000

Step#2: Calculations of f_s, which is the fraction of donor electrons transferred to cell mass

Culture pH	f_s
4.5	0.161
5.5	0.198
6.5	0.222
7.5	0.298
8.5	0.309

Fermentation time (h)	f_s
4	0.154
6	0.185
8	0.227
10	0.299
12	0.344

Culture temperature (°C)	f_s
15	0.212
20	0.226
25	0.235
30	0.244
35	0.253

S/X	f_s
0.5	0.253
1.0	0.250
1.5	0.262
2.0	0.275
2.5	0.283

Step#3: Calculations of R_a, which is the half reaction for mixed electron acceptors

Culture pH	Left hand side				Right hand side					
	CO_2	HCO_3^-	H^+	e^-	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	H_2	H_2O
4.5	0.124	0.071	1.000	1.000	0.048	0.015	0.004	0.003	0.106	0.285
5.5	0.116	0.068	1.000	1.000	0.047	0.014	0.004	0.002	0.128	0.270
6.5	0.117	0.068	1.000	1.000	0.047	0.014	0.005	0.002	0.124	0.273
7.5	0.129	0.075	1.000	1.000	0.050	0.015	0.007	0.003	0.085	0.300
8.5	0.131	0.075	1.000	1.000	0.048	0.015	0.008	0.004	0.080	0.304

Fermentation time (h)	Left hand side				Right hand side					
	CO_2	HCO_3^-	H^+	e^-	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	H_2	H_2O
4	0.123	0.072	1.000	1.000	0.048	0.014	0.006	0.004	0.105	0.286
6	0.121	0.070	1.000	1.000	0.049	0.015	0.005	0.001	0.114	0.281
8	0.118	0.068	1.000	1.000	0.049	0.015	0.004	0.001	0.123	0.273
10	0.118	0.069	1.000	1.000	0.049	0.014	0.005	0.001	0.120	0.277
12	0.118	0.067	1.000	1.000	0.044	0.014	0.007	0.002	0.124	0.273

Culture temperature (°C)	Left hand side				Right hand side					
	CO_2	HCO_3^-	H^+	e^-	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	H_2	H_2O
15	0.123	0.070	1.000	1.000	0.044	0.014	0.006	0.005	0.109	0.283
20	0.119	0.069	1.000	1.000	0.048	0.015	0.005	0.001	0.119	0.276
25	0.118	0.067	1.000	1.000	0.045	0.014	0.005	0.003	0.125	0.271
30	0.118	0.067	1.000	1.000	0.045	0.015	0.004	0.003	0.127	0.270
35	0.116	0.067	1.000	1.000	0.048	0.015	0.004	0.001	0.132	0.267

S/X	Left hand side				Right hand side					
	CO_2	HCO_3^-	H^+	e^-	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	H_2	H_2O
0.5	0.111	0.065	1.000	1.000	0.047	0.014	0.002	0.002	0.145	0.258
1.0	0.117	0.068	1.000	1.000	0.049	0.015	0.003	0.001	0.129	0.270
1.5	0.132	0.072	1.000	1.000	0.046	0.017	0.005	0.003	0.087	0.298
2.0	0.137	0.077	1.000	1.000	0.053	0.018	0.006	0.000	0.066	0.315
2.5	0.142	0.081	1.000	1.000	0.055	0.017	0.005	0.003	0.049	0.327

Step#4: Calculations of $f_e \cdot R_a$

Culture pH	Left hand side				Right hand side					
	CO_2	HCO_3^-	H^+	e^-	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	H_2	H_2O
4.5	0.104	0.059	0.839	0.839	0.040	0.013	0.004	0.003	0.089	0.239
5.5	0.093	0.054	0.802	0.802	0.038	0.011	0.003	0.002	0.103	0.216
6.5	0.091	0.053	0.778	0.778	0.037	0.011	0.004	0.002	0.097	0.212
7.5	0.091	0.053	0.702	0.702	0.035	0.010	0.005	0.002	0.060	0.211
8.5	0.090	0.052	0.691	0.691	0.033	0.010	0.006	0.003	0.055	0.210

Fermentation time (h)	Left hand side				Right hand side					
	CO_2	HCO_3^-	H^+	e^-	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	H_2	H_2O
4	0.104	0.061	0.846	0.846	0.040	0.012	0.005	0.004	0.089	0.242
6	0.098	0.057	0.815	0.815	0.040	0.012	0.004	0.001	0.093	0.229
8	0.091	0.053	0.773	0.773	0.038	0.012	0.003	0.001	0.095	0.211
10	0.083	0.049	0.701	0.701	0.034	0.010	0.004	0.000	0.084	0.194
12	0.077	0.044	0.656	0.656	0.029	0.009	0.004	0.001	0.081	0.179

Culture temperature (°C)	Left hand side				Right hand side					
	CO_2	HCO_3^-	H^+	e^-	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	H_2	H_2O
15	0.097	0.055	0.788	0.788	0.035	0.011	0.005	0.004	0.086	0.223
20	0.092	0.053	0.774	0.774	0.037	0.012	0.004	0.001	0.092	0.214
25	0.090	0.051	0.765	0.765	0.034	0.011	0.004	0.002	0.096	0.208
30	0.089	0.050	0.756	0.756	0.034	0.011	0.003	0.002	0.096	0.204
35	0.086	0.050	0.747	0.747	0.036	0.011	0.003	0.001	0.098	0.200

S/X	Left hand side				Right hand side					
	CO_2	HCO_3^-	H^+	e^-	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	H_2	H_2O
0.5	0.083	0.049	0.747	0.747	0.035	0.010	0.002	0.001	0.108	0.193
1.0	0.088	0.051	0.750	0.750	0.037	0.011	0.002	0.001	0.096	0.202
1.5	0.097	0.053	0.738	0.738	0.034	0.013	0.004	0.002	0.064	0.220
2.0	0.099	0.056	0.725	0.725	0.038	0.013	0.005	0.000	0.048	0.228
2.5	0.102	0.058	0.717	0.717	0.040	0.013	0.004	0.002	0.035	0.234

Step#5: Calculations of $f_s \cdot R_c$

Culture pH	Left hand side					Right hand side	
	CO_2	HCO_3^-	H^+	e^-	NH_4^+	$C_5H_7O_2N$	H_2O
4.5	0.032	0.008	0.161	0.161	0.008	0.008	0.072
5.5	0.040	0.010	0.198	0.198	0.010	0.010	0.089
6.5	0.044	0.011	0.222	0.222	0.011	0.011	0.100
7.5	0.060	0.015	0.298	0.298	0.015	0.015	0.134
8.5	0.062	0.015	0.309	0.309	0.015	0.015	0.139

Fermentation time (h)	Left hand side					Right hand side	
	CO_2	HCO_3^-	H^+	e^-	NH_4^+	$C_5H_7O_2N$	H_2O
4	0.031	0.008	0.154	0.154	0.008	0.008	0.069
6	0.037	0.009	0.185	0.185	0.009	0.009	0.083
8	0.045	0.011	0.227	0.227	0.011	0.011	0.102
10	0.060	0.015	0.299	0.299	0.015	0.015	0.135
12	0.069	0.017	0.344	0.344	0.017	0.017	0.155

Culture temperature (°C)	Left hand side					Right hand side	
	CO_2	HCO_3^-	H^+	e^-	NH_4^+	$C_5H_7O_2N$	H_2O
15	0.042	0.011	0.212	0.212	0.011	0.011	0.095
20	0.045	0.011	0.226	0.226	0.011	0.011	0.102
25	0.047	0.012	0.235	0.235	0.012	0.012	0.106
30	0.049	0.012	0.244	0.244	0.012	0.012	0.110
35	0.051	0.013	0.253	0.253	0.013	0.013	0.114

S/X	Left hand side					Right hand side	
	CO_2	HCO_3^-	H^+	e^-	NH_4^+	$C_5H_7O_2N$	H_2O
0.5	0.051	0.013	0.253	0.253	0.013	0.013	0.114
1.0	0.050	0.013	0.250	0.250	0.013	0.013	0.113
1.5	0.052	0.013	0.262	0.262	0.013	0.013	0.118
2.0	0.055	0.014	0.275	0.275	0.014	0.014	0.124
2.5	0.057	0.014	0.283	0.283	0.014	0.014	0.128

Step#6: Calculations of $f_e \cdot R_a + f_s \cdot R_c - R_d$

Culture pH	Left hand side			Right hand side							
	$C_6H_{12}O_6$	HCO_3^-	NH_4^+	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	H_2	CO_2	$C_5H_7O_2N$	H_2O
4.5	0.042	0.067	0.008	0.040	0.013	0.004	0.003	0.089	0.114	0.008	0.062
5.5	0.042	0.064	0.010	0.038	0.011	0.003	0.002	0.103	0.117	0.010	0.055
6.5	0.042	0.064	0.011	0.037	0.011	0.004	0.002	0.097	0.114	0.011	0.062
7.5	0.042	0.067	0.015	0.035	0.010	0.005	0.002	0.060	0.100	0.015	0.095
8.5	0.042	0.067	0.015	0.033	0.010	0.006	0.003	0.055	0.098	0.015	0.099

Fermentation time (h)	Left hand side			Right hand side							
	$C_6H_{12}O_6$	HCO_3^-	NH_4^+	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	H_2	CO_2	$C_5H_7O_2N$	H_2O
4	0.042	0.068	0.008	0.040	0.012	0.005	0.004	0.089	0.115	0.008	0.061
6	0.042	0.066	0.009	0.040	0.012	0.004	0.001	0.093	0.115	0.009	0.062
8	0.042	0.064	0.011	0.038	0.012	0.003	0.001	0.095	0.113	0.011	0.064
10	0.042	0.064	0.015	0.034	0.010	0.004	0.000	0.084	0.107	0.015	0.078
12	0.042	0.061	0.017	0.029	0.009	0.004	0.001	0.081	0.104	0.017	0.084

Culture temperature (°C)	Left hand side			Right hand side							
	$C_6H_{12}O_6$	HCO_3^-	NH_4^+	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	H_2	CO_2	$C_5H_7O_2N$	H_2O
15	0.042	0.066	0.011	0.035	0.011	0.005	0.004	0.086	0.111	0.011	0.068
20	0.042	0.064	0.011	0.037	0.012	0.004	0.001	0.092	0.112	0.011	0.066
25	0.042	0.063	0.012	0.034	0.011	0.004	0.002	0.096	0.113	0.012	0.063
30	0.042	0.063	0.012	0.034	0.011	0.003	0.002	0.096	0.112	0.012	0.064
35	0.042	0.062	0.013	0.036	0.011	0.003	0.001	0.098	0.113	0.013	0.064

S/X	Left hand side			Right hand side							
	$C_6H_{12}O_6$	HCO_3^-	NH_4^+	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	H_2	CO_2	$C_5H_7O_2N$	H_2O
0.5	0.042	0.061	0.013	0.035	0.010	0.002	0.001	0.108	0.116	0.013	0.056
1.0	0.042	0.063	0.013	0.037	0.011	0.002	0.001	0.096	0.112	0.013	0.065
1.5	0.042	0.066	0.013	0.034	0.013	0.004	0.002	0.064	0.100	0.013	0.088
2.0	0.042	0.070	0.014	0.038	0.013	0.005	0.000	0.048	0.096	0.014	0.102
2.5	0.042	0.072	0.014	0.040	0.013	0.004	0.002	0.035	0.092	0.014	0.112

Step#7: Overall reaction $R_{overall} = f_e \cdot R_a + f_s \cdot R_c - R_d$. (HYs are presented in bold)

Culture pH	Left hand side		Right hand side								
	$C_6H_{12}O_6$	NH_4^+	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	**H_2 (HY)**	CO_2	$C_5H_7O_2N$	H_2O	H^+
4.5	1.000	0.193	0.971	0.308	0.085	0.063	**2.138**	1.111	0.193	-0.140	1.620
5.5	1.000	0.238	0.910	0.265	0.083	0.047	**2.465**	1.274	0.238	-0.213	1.543
6.5	1.000	0.267	0.883	0.261	0.087	0.044	**2.317**	1.202	0.267	-0.054	1.542
7.5	1.000	0.357	0.845	0.249	0.110	0.059	**1.436**	0.773	0.357	0.657	1.620
8.5	1.000	0.371	0.796	0.243	0.138	0.063	**1.327**	0.732	0.371	0.763	1.611

Fermentation time (h)	Left hand side		Right hand side								
	$C_6H_{12}O_6$	NH_4^+	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	**H_2 (HY)**	CO_2	$C_5H_7O_2N$	H_2O	H^+
4	1.000	0.185	0.965	0.279	0.121	0.087	**2.134**	1.128	0.185	-0.174	1.637
6	1.000	0.222	0.962	0.287	0.098	0.025	**2.221**	1.159	0.222	-0.108	1.594
8	1.000	0.272	0.903	0.280	0.069	0.016	**2.291**	1.180	0.272	-0.015	1.540
10	1.000	0.359	0.824	0.240	0.092	0.009	**2.011**	1.051	0.359	0.359	1.525
12	1.000	0.412	0.701	0.222	0.103	0.030	**1.946**	1.025	0.412	0.538	1.469

Culture temperature (°C)	Left hand side		Right hand side								
	$C_6H_{12}O_6$	NH_4^+	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	**H_2 (HY)**	CO_2	$C_5H_7O_2N$	H_2O	H^+
15	1.000	0.255	0.841	0.265	0.120	0.091	**2.052**	1.086	0.255	0.062	1.572
20	1.000	0.271	0.887	0.276	0.090	0.023	**2.213**	1.151	0.271	0.028	1.547
25	1.000	0.282	0.821	0.263	0.089	0.059	**2.294**	1.192	0.282	0.006	1.513
30	1.000	0.293	0.819	0.267	0.073	0.052	**2.304**	1.188	0.293	0.030	1.504
35	1.000	0.303	0.852	0.266	0.064	0.014	**2.364**	1.214	0.303	0.027	1.499

S/X	Left hand side		Right hand side								
	$C_6H_{12}O_6$	NH_4^+	CH_3COO^- (Acetate)	$CH_3CH_2CH_2COO^-$ (Butyrate)	$CH_3CH_2COO^-$ (Propionate)	$CH_3CHOHCOO^-$ (Lactate)	H_2 (HY)	CO_2	$C_5H_7O_2N$	H_2O	H^+
0.5	1.000	0.303	0.847	0.252	0.040	0.031	**2.598**	1.319	0.303	-0.118	1.473
1.0	1.000	0.300	0.882	0.274	0.048	0.013	**2.316**	1.182	0.300	0.041	1.517
1.5	1.000	0.314	0.817	0.305	0.092	0.059	**1.546**	0.819	0.314	0.520	1.587
2.0	1.000	0.331	0.917	0.307	0.110	0.008	**1.145**	0.627	0.331	0.781	1.672
2.5	1.000	0.340	0.950	0.301	0.091	0.051	**0.847**	0.469	0.340	0.943	1.733

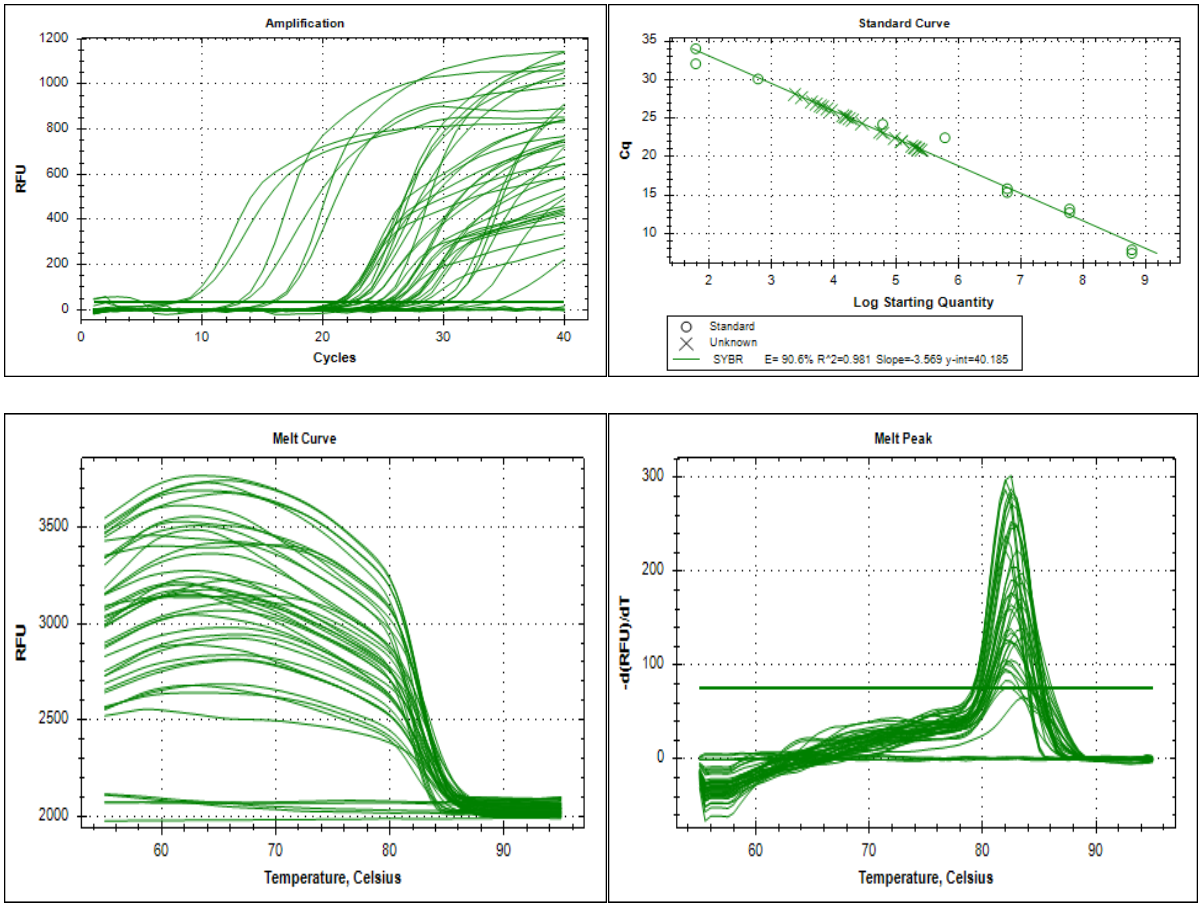


Figure 11 Quantitative polymerase chain reaction results for *Clostridium* amplification.

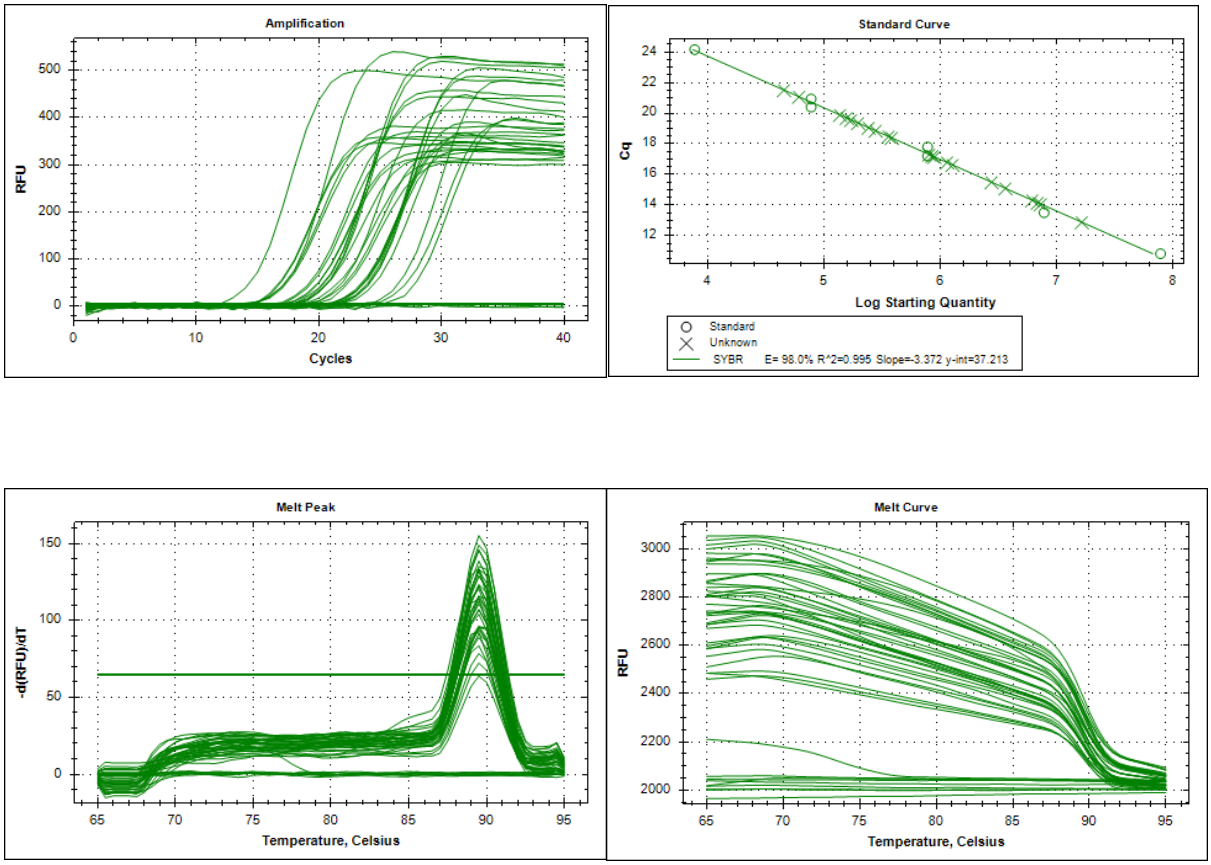


Figure 12 Quantitative polymerase chain reaction standard for *Enterobacteriaceae* amplification.

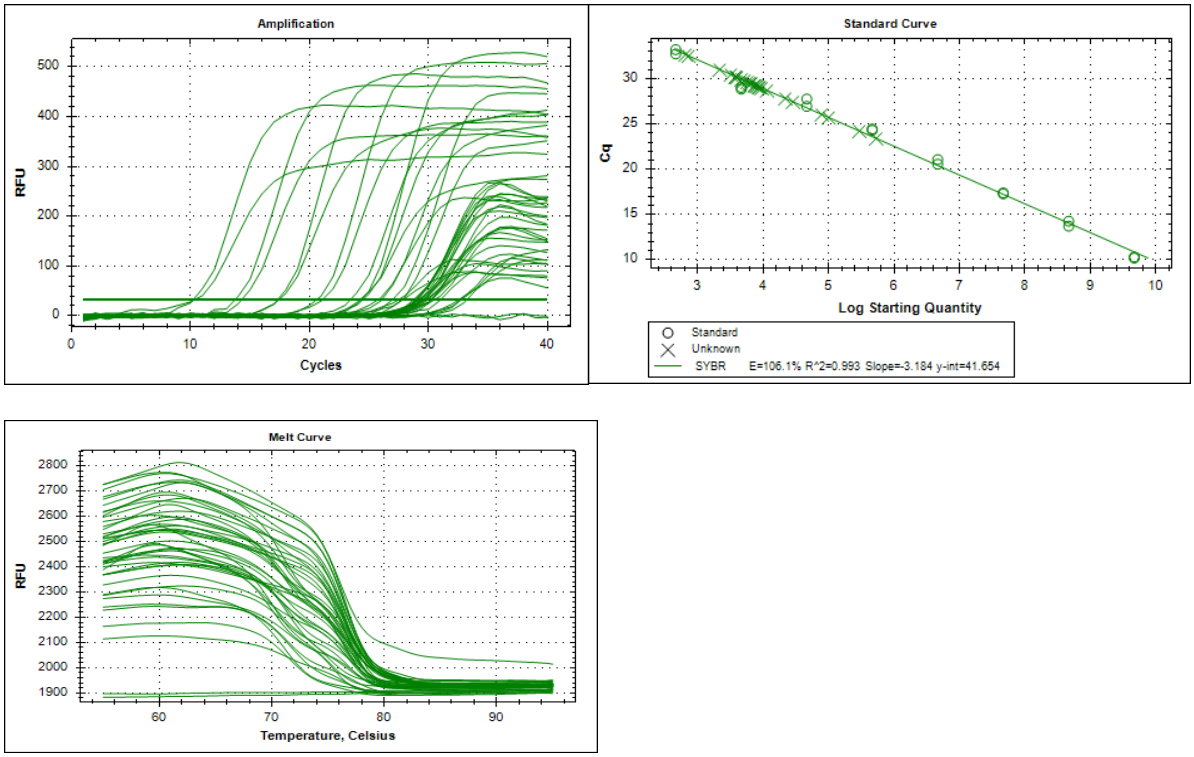


Figure 13 Quantitative polymerase chain reaction results for *Hydrogenase* amplification.

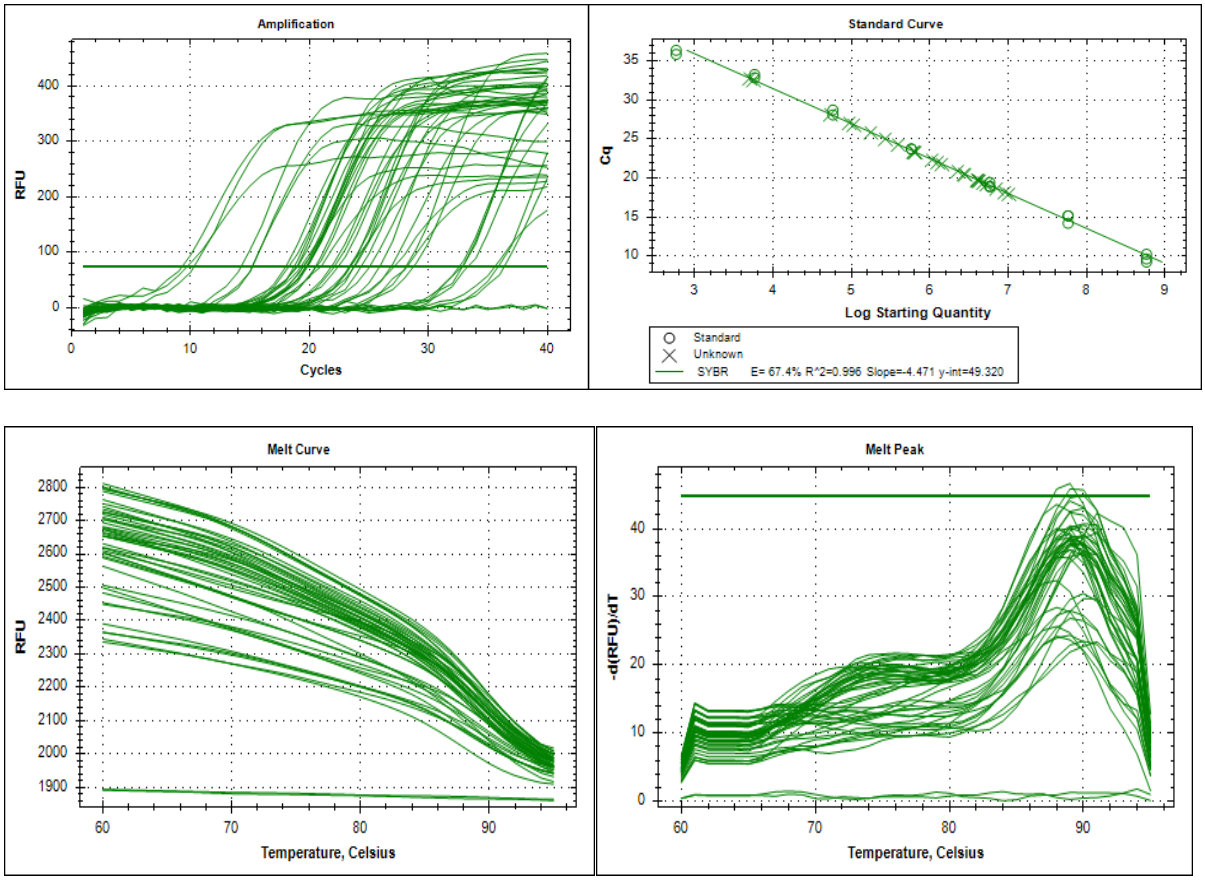


Figure 14. Quantitative polymerase chain reaction results for *EUB* amplification.

Appendix, section 4.2.1 Inoculum characterization

Determination of total suspended solids (TSS) in biomass

Total suspended solids in biomass were determined by first placing marked crucibles in a furnace at 575 ± 250°C to ignite all previous materials present in the crucible. The crucibles were removed and cooled down to room temperature in a desiccator and recorded the weight. The cycle was repeated (of drying or igniting, cooling, desiccating, and weighing) until a constant weight was obtained. A glass fibre filter paper was used to filter 15 ml sample volume (2.5 and 200 mg dried residue) in a filtration unit. After filtration the was filter removed from filtration apparatus and transfer to a crucible and weight. The combination of filter and crucible was transferred to an oven to dry for at 1

h at 105°C, and cooled down in a desiccator to balance temperature, and weigh. The cycle was repeated until constant weight was obtained.

$$mg\ Total\ suspended\ solids/L = \frac{(A-B)\ X\ \ 1000}{Sample\ volume, ml}$$

where: A = weight of filter + dried residue, mg, and B = weight of filter, mg.

Determination of volatile solids (VS) in biomass

The residues produced by TSS method was ignited in a muffle furnace at a temperature of 550°C. a blank glass fibre filter along with samples was also ignited. The cycle was repeated until constant weight was obtained. The dish or filter disk was cooled down partially in air until most of the heat has been dissipated and was therefore transferred to a desiccator for final cooling in a dry atmosphere. The Weight of the dish or disk was recorded as soon as it has cooled to balance temperature.

$$mg\ Total\ volatile\ solids/L = \frac{(A-B)\ X\ \ 1000}{Sample\ volume, ml}$$

$$mg\ fixed\ solids/L = \frac{(B-C)\ X\ \ 1000}{Sample\ volume, ml}$$

where: A = weight of residue + dish before ignition, mg, B = weight of residue + dish or filter after ignition, mg, and C = weight of dish or filter, mg

pH

pH is defined as a negative logarithm of the hydrogen ion concentration representing the degree of acidity and alkalinity of H_2O. The pH of the samples was determined using a pH meter.

COD

The COD concentration in the wastewater was determined by colorimetric standard according to the standard method. A heating block digester heating block digester (Milestone S.R.L.) was first used to digest the samples plus a reagent blank in COD vials at 150°C for 2 h. The digested samples and blank were cooled down and filtered using a 0.45 µm pore size filter. The Aquakem Gallery discrete autoanalyser (Thermo Scientific, UK) was used to measure COD concentration.

APPENDIX THREE: (Chapter V)

Gram stain

A smear from nutrient agar plate was prepared and placed on clean glass slide. The slide was fixed burnsen burner flame untile dry. Crystal violet was applied for 20 seconds, iodine was added, the mordant to fix crystal violet for 20 seconds. Alcohol was applied as decolorizing agent to remove the primary stain, washed and applied safranin followed by the counterstain, for 20 seconds. The slide was rinsed and allowed to air dry. Viewed with light microscope.

Nutrient agar

Composition of Nutrient Agar

- 0.5% Peptone.
- 0.3% beef extract/yeast extract
- 1.5% agar.
- 0.5% NaCl
- Distilled water
- pH (7.4) at 25 °C

APPENDIX FOUR: (Chapter VI)

Table cost $ /kg of H2 produced

Substrate	Inoculum	Condition	Value ($ /kg of H_2 produced)	Reference
Agricultural product, organic wastes and organic acids	Fermentative and photosynthetic bacteria	pH 4.4-6.5	2.57 2.83	(Nikolaidis and Poullikkas, 2017)
Corn Stover	*Clostridium thermocellum*	HRT 75hr Temp 55°C	(2015) 50 (2025) 8.56	(Randolph and Studer, 2017)
Sugar cane	*Clostridium* spp, *Klebsiella* spp. *and Enterobacter* spp.	S/X 8–24 g/L HRT 24–96 hr. Temp 37°C pH 6.5	0.96	(Moodley, 2015)
Sulfur-iodine Calcium bromine - iron		Temp 50-730 °C	1.30	(Penner, 2006)
coffee	*Thermoanaerobacterium Thermosaccharolyticum*	Particle size 1-2cm Moisture10-20% Temp 21- 800 °C	1.6 and 2.7	(García and Cardona) (García et al., 2017)
Glucose organic metabolites	*Enterobacter cloacae* strain DM11 *Rhodobacter sphaeroides* strain		2.74	(Bharathiraja et al., 2016, Khanna and Das, 2013)
Water	Green algae		1.42	(Nikolaidis and Poullikkas, 2017)
Pinus patula plant	Air, water or oxygen-gasifying agent	Air 0.25 Kg Air/kg Biomass particle size of 1-2 cm and a moisture content of 20%	1,57	(García and Cardona)
Water	Green algae		10	(Hallenbeck and Benemann, 2002)
Wind energy water			8 – 10	(Das, 2014)
Water	Green algae		13.5	(Sen et al., 2008)
Natural gas (via steam reforming)			4 – 5	(Das, 2014)
glucose			18.7	(Elbeshbishy et al., 2017)