OPTIMIZATION OF SELECTED FERMENTATION PARAMETERS FOR MICROBIAL BIOHYDROGEN PRODUCTION USING FOOD WASTE AS THE MAJOR SUBSTRATE

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ABSTRACT

The energy crisis and current environmental degradation are the two vital issues for global sustainable development. Hydrogen is seen as the energy of the future; looking at the fluctuating price of oil and other natural gases prices, on top of the increasing global awareness of increasing carbon dioxide level. This carbon dioxide level is associated to global warming, acid rain and other disastrous phenomenon. Hydrogen is a sustainable energy source with minimal use of hydrocarbon. These, plus the high energy yield of 122 kJ/g makes hydrogen an attractive alternative to fossil fuels (Guo *et al*, 2010)

The objective of this study was to find the optimum condition for anaerobic co-digestion of food waste and sewage sludge and hydrogen (H_2) production. The selected parameters for optimization of hydrogen production (e.g. temperature, initial pH, inoculum size) were analysed using Response Surface Methodology with Full Factorial Design. Two types of substrates were tested; food waste as a sole substrate (Production 1) and food waste mixed with palm oil mill effluent (POME) at volume ratio 1:1 (Production 2).

The optimized conditions for both Production 1 and Production 2 were pH 4.5, temperature of 35°C and inoculum size of 20%, (v/v) with maximum predicted cumulative hydrogen production (MPCHP) of 0.22 ml hydrogen /ml substrate and 0.26 ml hydrogen /ml substrate, respectively.

Subsequent verification experiments at optimal parameter values yielded cumulative hydrogen of 0.28 ml hydrogen /ml substrate for Production 1 and 0.33 ml H_2/ml substrate for Production 2.

ABSTRAK

Krisis tenaga dan pencemaran alam sekitar pada hari ini merupakan dua perkara mustahak untuk pembangunan terperinci global. Hidrogen dilihat sebagai sumber tenaga masa hadapan; melihat pada naik turun harga minyak dan gas asli yang lain. Tambahan pula peningkatan kesedaran tentang kenaikan paras karbon dioksida dunia. Paras karbon dioksida ini berkait rapat dengan pemanasan global, hujan asid dan fenomena kemusnahan alam yang lain. Hidrogen ialah sumber tanaga yang boleh diperbaharui dengan penggunaan hidrokarbon yang minimum. Hidrogen juga mencatat penghasilan tenaga yang tinggi iaitu sebanyak 122 kJ/g, menjadikannya alternatif yang menarik kepada minyak fosil (Guo *et al*, 2010).

Objektif kajian ini adalah untuk mencari keadaan yang paling optimum untuk penghadaman bersama sisa makanan dan sisa kumbahan serta penghasilan hidrogen. Parameter yang dipilih untuk mengoptimumkan penghasilan hidrogen (suhu, pH awalan dan saiz inokulum) dianalisa menggunakan *Response Surface Methodology* dengan *Full Factorial Design.* Terdapat dua jenis substrat yang diuji; sisa makanan sahaja sebagai substrat (Produksi 1) dan sisa makanan dicampur dengan sisa buangan kilang kelapa sawit pada nisbah isipadu 1:1 (Produksi 2).

Keadaan optimum untuk kedua-dua Produksi 1 dan Produksi 2 adalah pH awalan 4.5, suhu 35°C dan saiz inokulum 20% (isipadu/ isipadu) dengan anggaran penghasilan maksimum hidrogen kumulatif masing-masing sebanyak 0.22 ml hidrogen/ ml substrat dan 0.26 ml hidrogen/ ml substrat.

Kajian pengesahan seterusnya dilakukan dengan menggunakan nilai pada keadaan optimum. Kajian ini mencatatkan penghasilan hidrogen kumulatif 0.28 ml hidrogen/ ml substrat untuk Produksi 1 dan 0.33 ml hidrogen/ ml substrat untuk Produksi 2.

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CONTENTS

FROM WASTE

ABBREVIATIONS

BOD	Biological oxygen demand
COD	Chemical oxygen demand
g	Gram
GC	Gas chromatography
h	Hour
H_2	Hydrogen
min	Minute
ml	Millilitre
POME	Palm Oil Mill Effluent

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OBJECTIVES OF STUDY

I. To determine effect of temperature, inoculum size and initial pH on microbial hydrogen production

II. To study the effect of sparging to produce a fully anaerobic condition for microbial hydrogen production

III. To compare biohydrogen production in systems with and without palm oil mill effluent (POME) supplement

IV. To apply Response Surface Methodology (RSM) in optimizing the system for microbial hydrogen production

1.0 INTRODUCTION

1.1 Hydrogen as an Energy Source

Fossil fuels are quickly depleting and the fact that it causes pollution from emission of carbon dioxide (CO_2), carbon monoxide (CO), sulphur monoxide (SO), nitrogen oxide (NO), among others increase the pressing needs to develop non-polluting and renewable energy sources. Table 1.1 shows the world oil demand and supply forecasted until 2030. It is obvious that the demand for oil in the major countries like United States, Europe, China, and India will keep increasing and the supply will be insufficient over the years.

Table 1.1: World primary oil demand and supply in million barrels per day (World Energy Outlook, 2006).

Demand	1980	2004	2005	2010	2015	2030
United States	17.4	20.5	20.6	21.6	23.1	25.0
Europe	14.7	14.5	14.4	14.9	15.4	15.4
China	1.9	6.5	6.6	8.4	10.0	15.3
India	0.7	2.6	2.6	3.2	3.7	5.4

Supply	1980	2004	2005	2010	2015	2030
United States	8.7	5.8	5.1	5.3	5.0	4.0
Europe	2.4	6.2	4.8	3.8	2.9	1.5
China	2.1	3.2	3.6	3.8	3.7	2.8
India	0.2	0.6	0.7	0.8	0.8	0.6

The energy demand and current environmental degradation are the two vital issues for global sustainable development. Hydrogen (H_2) is seen as the energy of the

future; looking at the fluctuating price of oil and other natural gases prices, on top of the increasing global awareness of increasing carbon dioxide level. This carbon dioxide level is associated with global warming, acid rain and other disastrous phenomenon. Hydrogen is a sustainable energy source with minimal use of hydrocarbon. These, plus the high energy yield of 122 kJ/g makes hydrogen an attractive alternative to fossil fuels (Guo, Trably, Latrille, Carrere & Steyer, 2010)

Demand on hydrogen is not just limited for use as an energy source only, hydrogen gas is a widely used as feedstock for production of chemicals, hydrogenation of fats and oils in food industry, production of electronic devices, processing steel and also for desulfurization and re-formulation of gasoline in refineries.

Conventional hydrogen gas production methods are steam reforming of methane (SRM), and other hydrocarbons (SRH), non-catalytic partial oxidation of fossil fuels (POX) and autothermal reforming which combines SRM and POX. These processes require high energy level and temperatures (more than 850°C). Among other methods that have been developed for improvement are the membrane processes, selective oxidation of methane and oxidative dehydrogenation (Kapdan & Kargi, 2006).

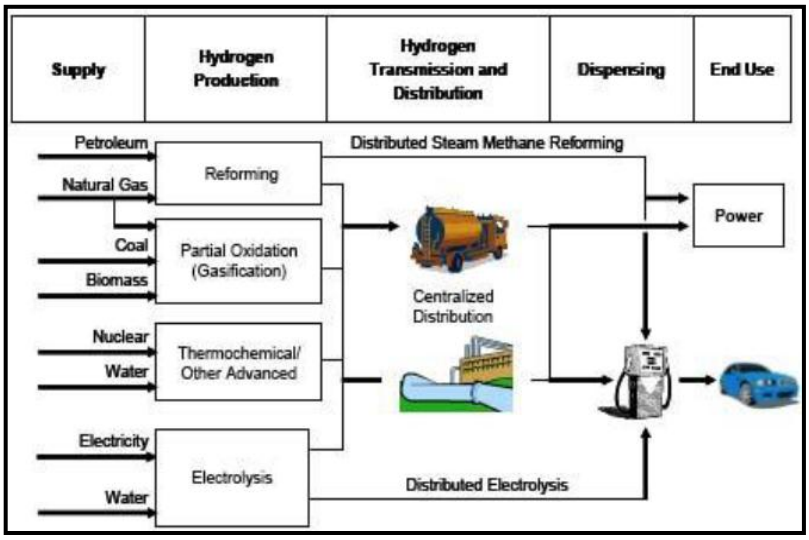

Figure 1.1: A simplified examples of hydrogen production pathways (Lipman, 2011).

Biohydrogen production is a viable alternative to the other methods of hydrogen gas production. Sustainable development and waste minimization issues cause biohydrogen gas production from renewable sources to receive a lot of attention in recent years. Biohydrogen production as a green technology can achieved by anaerobic and photosynthetic microorganisms using carbohydrate rich and non-toxic raw materials. Under anaerobic conditions, hydrogen is produced as a by-product during conversion of organic wastes into organic acids which are then used for methane generation. Acidogenic phase of anaerobic digestion of wastes can be manipulated to improve hydrogen production.

Photosynthetic processes include algae and other photosynthetic microorganisms, which use carbon dioxide and water for hydrogen gas production. Some photo-heterotrophic bacteria utilize organic acids such as acetic, lactic and butyric acids to produce H_2 and CO_2. The advantages of the photosynthesis method are high hydrogen production and utilization of waste materials for the production. However, the rate of hydrogen production is low and the technology for this process needs further development (Levin, Islam, Cicek & Sparling, 2004).

Biohydrogen can be used directly in engines or in fuel cells for producing electricity. Hydrogen has high energy content per unit weight. Through oxidative combustion, water is the only by-product making biohydrogen a green energy source that produce minimal pollution to the environment. Hydrogen can also be used as fuel cells to directly generate electricity (Pan, Zhang, El-Mashad, Sun & Ying, 2008). Production of biohydrogen through fermentative process uses a wide range of organic substances and is technically simpler than photosynthetic processes.

Hydrogen is produced by both photosynthetic and chemosynthetic microorganisms. In the case of chemosynthetic microorganisms, members of the genus

Clostridium are well known for their high hydrogen evolution rate during anaerobic glucose degradation. Equation 1.1 shows production of hydrogen from hydrolysis of glucose.

$$C_6H_{12}O_6 + 6\,H_2O \rightarrow 6\,CO_2 + 12\,H_2 \qquad \text{(Equation 1.1)}$$

However, the bacteria also require metabolic energy for growth, which would limit the production of hydrogen from carbohydrate to about 4 mol/mol of hexose, reducing about 33% of the theoretical yield. (Doelle, 1994). Stoichiometrically, *Clostridium sp* can produce 2 moles of hydrogen with 1 mole of *n*-butyrate or 4 moles hydrogen with 2 moles of acetate from 1 mole of hexose (Kim, Han & Shin 2004).

1.2 Anaerobic Digestion

Anaerobic digestion is used mostly for the stabilization of solid composition. It is a biological process, in the absence of oxygen for the breakdown of the organic material by conversion to methane, carbon dioxide, biomass and inorganic products. It is a process found in many naturally occurring anoxic environments including watercourses, sediments or waterlogged soil. It can also be applied to a wide range of feed stocks including industrial and municipal wastewaters, agricultural waste, food industry waste and municipal waste (Ward *et al,* 2008).

In municipal solid waste treatment, anaerobic digestion is one of the options available, mainly because anaerobic digestion gives useful by-products such as compost and biogas. These by-products can be utilised as an energy source in developing countries. It only requires relatively small space and it helps to cut down the amount of greenhouse emissions compared to incineration or combustion, aerobic composting and land-filling.

Hydrolytic bacteria form a variety of reduced end-products from the fermentation of a given substrate. Questions have arisen about the metabolic features that actually control carbon and electron flow to produce a given reduced end-product during pure culture and mixed methanogenic cultures of hydrolytic bacteria.

Thermoanaerobium brockii is a thermophilic, hydrolytic bacterium that ferments glucose via the Embden-Meyerhof Parnas pathway. *T. brockii* is an atypical hetero-lactic acid bacterium because it forms molecular hydrogen (H_2), along with lactic acid and ethanol. The reduced end-products of glucose fermentation are enzymatically formed from pyruvate.

Anaerobic digestion of waste has three basic steps; hydrolysis, fermentation and methanogenesis.

1) Hydrolysis

Hydrolysis is the first step in anaerobic digestion where complex organics, such as proteins, carbohydrates and lipids, are converted to soluble organic compounds that can be hydrolyzed further to simple monomers through extracellular enzymes.

2) Fermentation

In fermentation, two processes are involved; acidogenesis and acetogenesis. Acidogenesis occur where soluble organics such as glucose, amino acids and fatty acids are degraded by acidogenic bacteria to volatile fatty acids (VFAs) and alcohols. After that, acetogenesis will proceed where conversion of VFAs to acetate, H_2 and CO_2 by acetogenic bacteria.

In the acidogenesis phase, complex molecules such as carbohydrates, lipids, and proteins are broken down into soluble compounds by hydrolytic enzymes. Examples of the hydrolytic enzymes are cellulases, hemicellulases, amylases,

lipases and proteases. The hydrolyzed compounds are fermented into volatile fatty acids (acetate, propionate, butyrate and lactate), neutral compounds (ethanol, methanol), ammonia, hydrogen and carbon dioxide.

Acetogenesis is one of the main reactions of this fermentation stage. The intermediary metabolites produced are later on metabolized to acetate, hydrogen and carbon dioxide gas by these three main groups of bacteria; homoacetogens, syntrophes and sulphoreductors. For the acetic acid production, the bacteria *Clostridium aceticum*, *Acetobacter woodii* and *Clostridium termoautotrophicum* are considered. *Clostridium* sp produces butyrate and acetate, Enterobacteria produce acetate and lactate, and hetero-fermentative bacteria will produce acetate, propionate, butyrate and valerate, among others.

3) Methanogenesis

Methanogenesis is the step where acetate, H_2 and CO_2 are converted to methane gas by methanogenic bacteria. Two groups of methanogenic bacteria are involved in methane production. The first one is aceticlastic methanogens that separate acetate into methane and carbon dioxide. The other one is a hydrogen-utilizing methanogens that use hydrogen as electron donor and CO_2 as the electron acceptor to produce methane

The production of biogas through anaerobic digestion has a lot of advantages compared to other forms of waste treatment, including:

- Less biomass is produced;
- Successful in treating wet mass with less than 40% dry matter;
- More effective pathogen removal;
- Minimal emission of odour as 99% of volatile compounds are oxidatively decomposed during combustion;

- Higher degree of compliance with many of national waste strategies implemented to reduce the amount of biodegradable waste entering landfill;
- The slurry produced is an improved fertilizer in terms of both its availability to plants and its rheology;
- The biogas can be a source of neutral carbon energy

According to Ward *et al* (2008), carbon dioxide released through natural mineralisation is considered neutral in greenhouse gas terms, as the carbon has been recently removed from the atmosphere by plant uptake, to be released again as part of the carbon cycle. Controlled anaerobic digestion of organic material is therefore environmentally beneficial since; it can contain the decomposition process in a sealed environment; the potentially damaging methane is prevented from entering the atmosphere. Subsequent burning of the gas will release carbon-neutral carbon dioxide back to the carbon cycle. Also, the energy gained from combustion of methane will displace fossil fuels, reducing the production of carbon dioxide that is not part of the recent carbon cycle.

1.3 Anaerobic Microorganisms

Anaerobic microorganisms can be either obligate anaerobes or facultative anaerobes. An obligate anaerobe is microorganism that lives in environment without oxygen. Even a little amount of oxygen can heavily harm or kill this microbe. A facultative anaerobe on the other hand flourish in oxygen-less environment but can also survive in the presence of oxygen as it has a supporting mechanism under this condition.

A facultative anaerobic organism usually generates adenine triphosphate (ATP) by aerobic respiration in the presence of oxygen but is also capable of switching to fermentation in absence of oxygen. In contrast, obligate anaerobes die in the presence

of oxygen. Examples of facultative anaerobic bacteria are *Staphylococcus* (Gram positive), *Escherichia coli* (Gram negative), and *Listeria* (Gram positive).

The concentrations of oxygen and fermentable material in the environment somehow affect the organism's use of aerobic respiration versus fermentation to derive energy. In brewer's yeast, the Pasteur shift is the observed cessation of oxygen consumption when fermentable sugar is supplied.

In a growing culture, the cell disfavours respiration due to more requirements for production, when there is sufficient fermentable substrate available, regardless of the energy output per mole of fermented material is far less than from respiration's complete oxidation of the same substrate. However, the rate of production of ATP in fermentation can be up to a 100 times faster than that of oxidative phosphorylation. Hence, tissues and organisms that require fast consumption of ATP preferentially use anaerobic glycolysis.

Under aerobic conditions, the molecule passes through glycolysis, and then enters the citric acid cycle, where it is completely oxidized. The electrons removed as NADH during glycolysis and the citric acid cycle are passed through an electron transport chain (ETC) to create a proton motive force, which later generates ATP.

On the other hand, under anaerobic conditions, the citric acid cycle cannot be utilized as it generates too much NAD. Thus, the cell undergoes fermentation. The ATP produced is less than glycolysis but cell will compensate for this by consuming more glucose in shorter time. This shift from slow aerobic to rapid anaerobic consumption of glucose was first noted by Pasteur, hence the name ‘Pasteur Shift’.

Figure 1.2 shows the allosteric regulations of glycolysis in the presence and absence of oxygen. When oxygen level is limited, full glucose oxidation will decrease,

followed by decrease in levels of production of ATP and citrate. The Pasteur Effect allows accelerated glycolysis to compensate for defective ATP production. However in the event of absolute absence of oxygen, the spike in level of fructose-1,6-bisP, ADP, AMP and inorganic phosphate causes a series of allosteric regulations, represented by the green arrows to accelerate glycolysis.

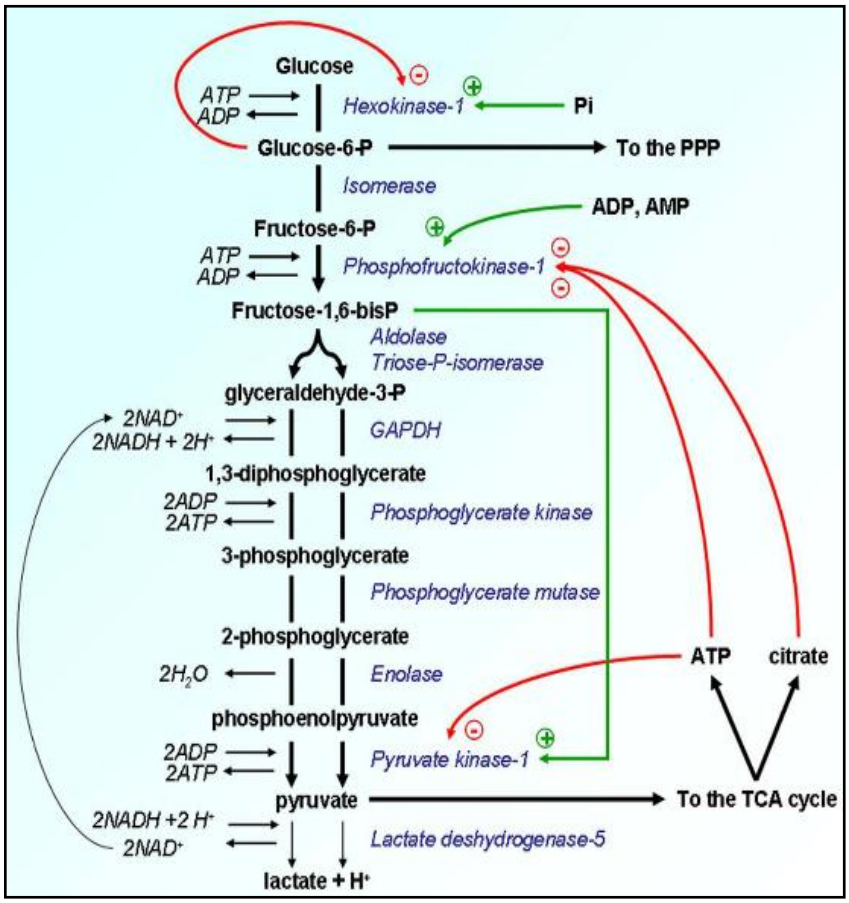

Figure 1.2: Allosteric regulations of glycolysis with respect to local oxygen (Porporato *et al*, 2011)

The sewage sludge is normally used as a source of microorganism mixed culture. This is often preferred than pure culture since it is simpler and easier to control. It also allows the use of broader sources of substrates and not to mention the potential for wastewater treatment. However, in a mixed culture system, hydrogen produced under anaerobic conditions may also be consumed by methanogens and homoacetogens to produce methane and acetic acid. Therefore, in order to obtain maximum yield of

hydrogen, the mixed culture, or sludge, need to undergo a pretreatment step to suppress as much as possible those hydrogen-consuming bacterias. Methods of pretreatments include mechanical, ultrasonic disintegration, alkali pretreatment, acidic pretreatment, heat pretreatment and thermo-chemical (Chong, Sabaratnam, Shirai & Hassan, 2009).

1.4 Chemical Oxygen Demand (COD)

The mass of an organic compound is not indicative for its COD. Hence, the expression "mass of organic material" in the case of COD does not really reflect the mass of the organic compounds, but rather the mass of oxygen required for a complete oxidation. So it can be concluded that the mass of consumed oxygen will always be equal to the mass of oxidised COD. The oxidation of organic material results it its transformation into stable, inorganic compounds such as carbon dioxide and water. Hence the mass of oxidised organic material (expressed as COD) can be measured directly by the consumption of oxygen required for this oxidation. This is the basis for respirometry; the study of biological processes by measuring the rate of oxygen consumption.

The theoretical COD per unit mass of $C_xH_yO_z$ is given by the Equation 1.2:

$$COD_t = 8_x(4_x + 1_y - 2_z)/(12_x + 1_y + 16_z)\ g\ COD\ g^{-1}\ C_xH_yO_z \qquad \text{(Equation 1.2)}$$

2.0 LITERATURE REVIEW

2.1 Domestic Waste and Management

There are a few types of waste such as domestic waste, industrial waste and building and construction waste. Domestic waste is the waste generated from everyday use of a household premise. Different type of waste will require different approach of management. This is to reduce the negative impact it may give to the environment. Figure 2.1 is a proof that food waste is the biggest contributor to solid waste.

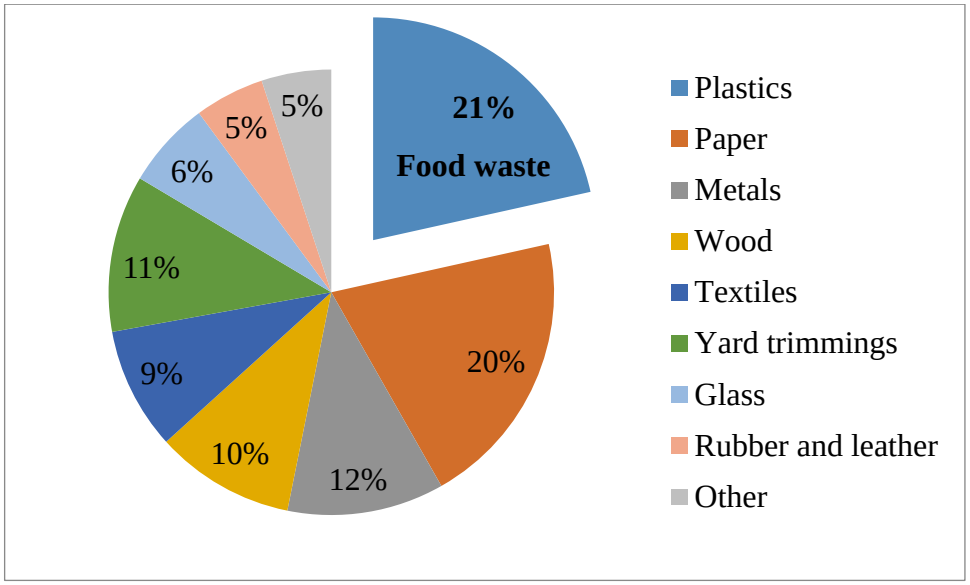

Figure 2.1: Data from the Municipal Solid Waste Characterization Report (US EPA, 2010).

Figure 2.2 shows the Lansink's ladder, which is the principle of EU policy in waste management. It was arranged in a decreasing manner from most preferred to least preferred. The first is prevention of waste production, by designing minimal waste production and design to enable beneficial use. Next is product recycling what is commonly called as "Reuse". The third step is material recycling for example plastic bottles, cans and papers. After that is incineration and the last one is disposal to landfill.

Landfill is considered as the last resort when the other options have been exhausted for waste management.

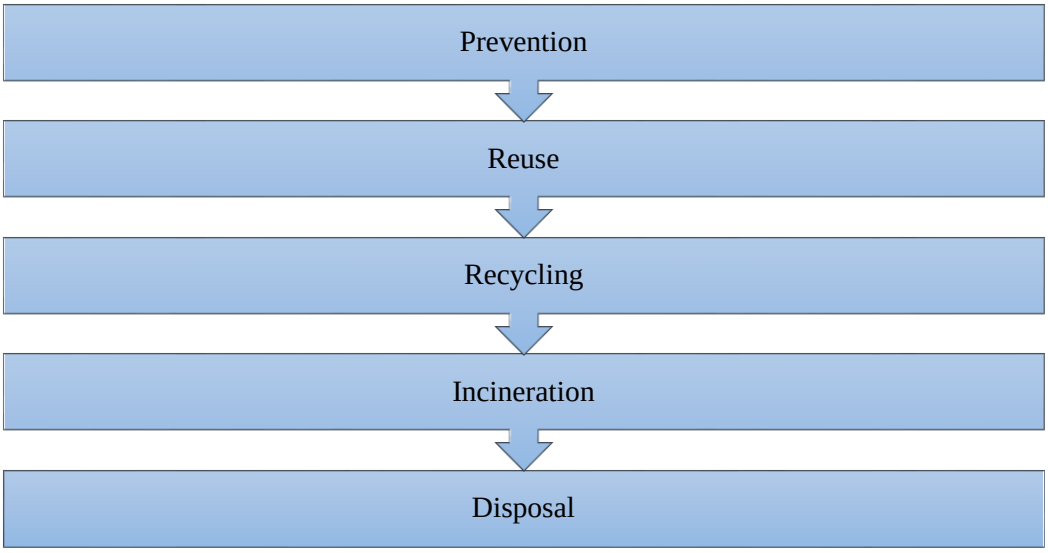

Figure 2.2: Ladder of Lansink

2.1.1 Incineration

Incineration is a process of controlled combustion for burning of wastes or residues containing combustible material. Pre-processing involves shredding, screening and magnetic separation to remove large and incombustible materials and recyclables. After incineration, the solid waste is mass-fired at 750°C to 1000°C in a combustion chamber which has a burning grate. The particulates and fly ash from exhaust steam will be removed by air pollution control equipment. Incineration system can be categorized on the basis of their air requirements; combustion, gasification and pyrolysis.

Advantages of incineration are that it helps to achieve maximum volume reduction instantly into biologically sterile product, approximately one-tenth of its pre-burnt volume and one-third of its pre-burnt weight. Incineration is a standard hygienic operation when compared to open burning. Waste incineration can be a source of

energy to produce steam for electric power generation, industrial process heating or hot water for district heating; thus conserving invaluable fossil fuel resources. Incineration requires minimal use of land and minimal burden on landfilling facilities. (Villanueva, 2007).

This method is favourable to countries with limited land area such as Singapore. In 2002, Singapore produces about 7200 tonne per day of solid waste. For a city with total land area of 682 km^2 only and population of 4.1 million people, Singapore has tremendous task of addressing waste disposal problem. 90% of the waste is incinerated in the four incineration plants constructed with energy recovery.

However, the incineration process also has some demerits. This process has higher costs towards capital, operation and maintenance and air pollution control equipments, and longer pay-back period. Negative public opinion towards incineration process may have limited its implementation in some countries. Their concern is air pollution caused by incineration that has adverse effect on health since it contains cancer causing dioxin, heavy metals, furans, halogenated organic compounds and other dangerous pollutants.

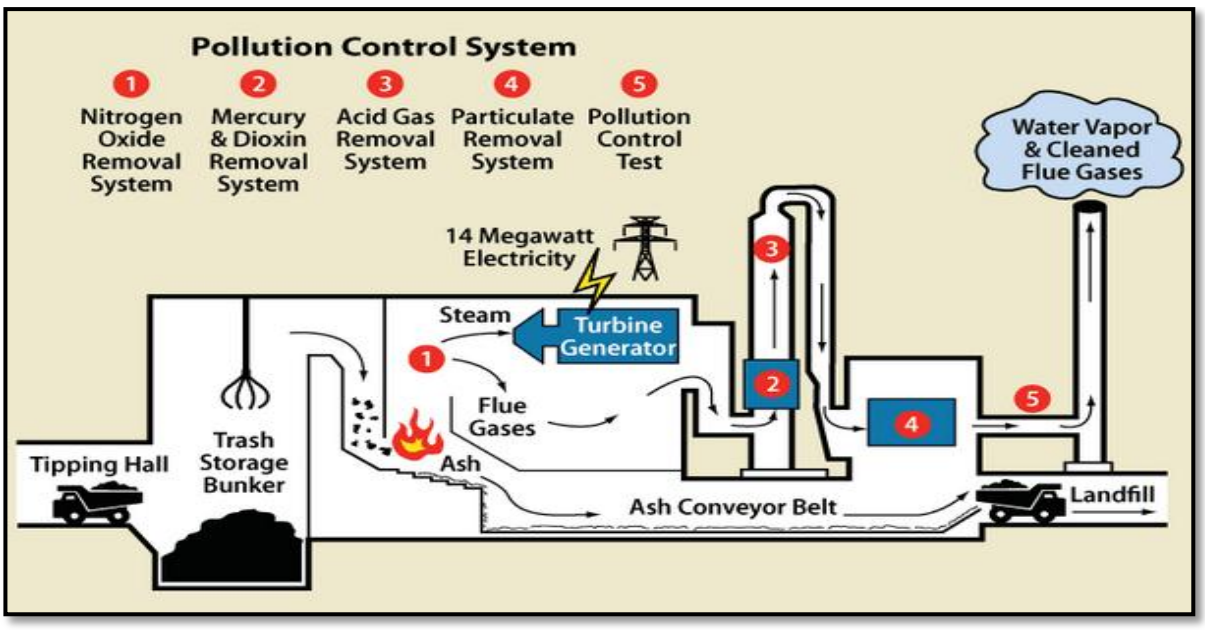

Figure 2.3: Waste to energy plant diagram (Energy Outlook, 2006)

Modern incinerator facilities can now be built with efficient combustion system equipped with sophisticated gas clean-up processes that can doubled as energy producers. This helps to reduce waste into inert residue with minimal pollution. Most developed nations in Europe, United States and Japan have already implemented stringent pollution emission limits from incineration plants.

2.1.2 Landfill

There are a few types of landfills; sanitary landfills, municipal solid waste landfills, industrial waste landfills and construction waste landfills. Construction waste landfills consist of the debris accumulated during construction, renovation and also demolition of buildings, roads and bridges. An industrial waste landfill is for nonhazardous solid waste associated with manufacturing and other industrial activities. A municipal solid waste landfill is for domestic waste and it uses a synthetic liner to isolate trash from the environment. A sanitary landfill has special geosynthetic and clay lining to prevent leachate from dissipating into the environment and contaminating the soil and ground water. Leachate consists of water and water-soluble compounds in the refuse; the water may be from rainfall or from the waste itself.

Landfill liners are used to create a barrier between the waste and the surrounding area. It also allows collection of leachate for treatment to reduce polluting effect before released to the environment (Hughes *et al*, 2005). Different disposal sites are available for different types of waste. The type of liner system required for each landfill will be determined by the potential threat posed by the waste.

Landfill gas is a strong greenhouse gas formed by anaerobic degradation of waste. Landfill gas has complex composition with the main components being methane, carbon dioxide, nitrogen and oxygen. There are also trace amount of carbon monoxide, hydrogen, hydrogen sulphide and other organic components.

Landfill gas can potentially cause explosions if it is located in urban areas. The gas produced can penetrate through soil and existing underground pipeline or cable systems into buildings nearby. A low atmospheric pressure will increase the risk of explosion since the low pressure can't effectively balance the gas pressure in the landfill. An example of this is the tragedy in Denmark on March 1991 where migrating gas from landfill caused fatal explosion. Odour problems related to landfill is often the result of inadequate topsoil cover and also indicate gas leak.

Extracting and burning of the landfill gas can be both environmentally and economically beneficial. In some countries it is a necessity to have some form of gas control as part of the management of landfills. The reasons behind it are to reduce risk of explosions, to reduce odour, to decrease carbon dioxide emission, to prevent groundwater pollution and to utilize the gas to generate fuel for revenue.

2.1.3 Waste management in Malaysia

In Malaysia, the most common method of waste management is disposal to landfills. There are a few incineration plants around the country but landfill disposal is more common. Municipal solid waste volumes generated within Selangor and the Kuala Lumpur are predicted to be 6650 tonne per day and expected to reach 12800 tonne per day by 2027. The amount of solid waste sent to dumping sites and operating and closed sites according to states are listed down in Table 2.1. The current method of disposal by landfill is becoming inadequate with increasing price and scarcity of land in urban areas. Developing new landfill outside of the city will only incur transportation cost and logistics.

This problem is best addressed by reducing the amount of waste to be disposed to the landfill. There are increasing awareness campaigns to recycle materials like plastic, glass and paper. Composting is also one way to do it though the amount is quite

small and seem less significant. Domestic waste can be incinerated for maximum reduction of mass to be disposed into landfills. Plus, there many waste-to-energy incineration plant being built around the world that can double-up as power supplier for the plant or other industries. However, the public generally have negative perception towards incineration due to its known air pollutant that may have adverse effect to health.

Table 2.1: Comparison of all solid waste in Malaysia (Jabatan Sisa Pepejal, 2010)

State	Waste collected (Metric tons/ day)	Number of operating sites	Number of closed dumping sites
Perlis	120	1	1
Kedah	1504	12	3
Penang	1800	1	2
Perak	1864	21	9
Pahang	1094	20	12
Selangor	3240	7	11
WP Kuala Lumpur	1950	1	7
Negeri Sembilan	1162	8	10
Melaka	906	2	5
Johor	2439	13	21
Kelantan	729	13	5
Terengganu	651	9	12
Sabah	1174	20	1
Sarawak	2001	51	12
Total per day	20633	179	111

The promise of green and renewable energy is great in the sense that it can utilize waste to be converted into energy. There have been a lot of studies done to produce energy biologically from biomass. Energy can be harvested in the form of gas such as methane and hydrogen. Food waste as a major contributor to municipal solid waste can be reduced by using it as a substrate for energy production. This has been done successfully on a lab scale by Ismail, Abdul Rahman, Abd Aziz, Ling & Hassan (2009) to produce hydrogen. The challenge is to plan and develop a plant for big scale waste to energy production. Feasibility studies need to be done since running a vast plant will require big capital cost for operation and production.

2.2 Palm Oil Mill Effluent

2.2.1 Palm oil industry in Malaysia

After Indonesia, Malaysia is the second largest exporter of palm oil in the world. The world's palm oil productions share is shown in Figure 2.4. In year 2010, the number of palm oil products exportation rocketed to 16.5 million tonnes. The crude palm oil production has increased from 7.8 million tonnes in 1995 to 17.56 million tonnes in 2009 (Ujang *et al*, 2012). Palm oil is assumed as one of the most important economic sources in the nation and contributed to the remarkable rise in the Malaysian's Gross domestic product (GDP).

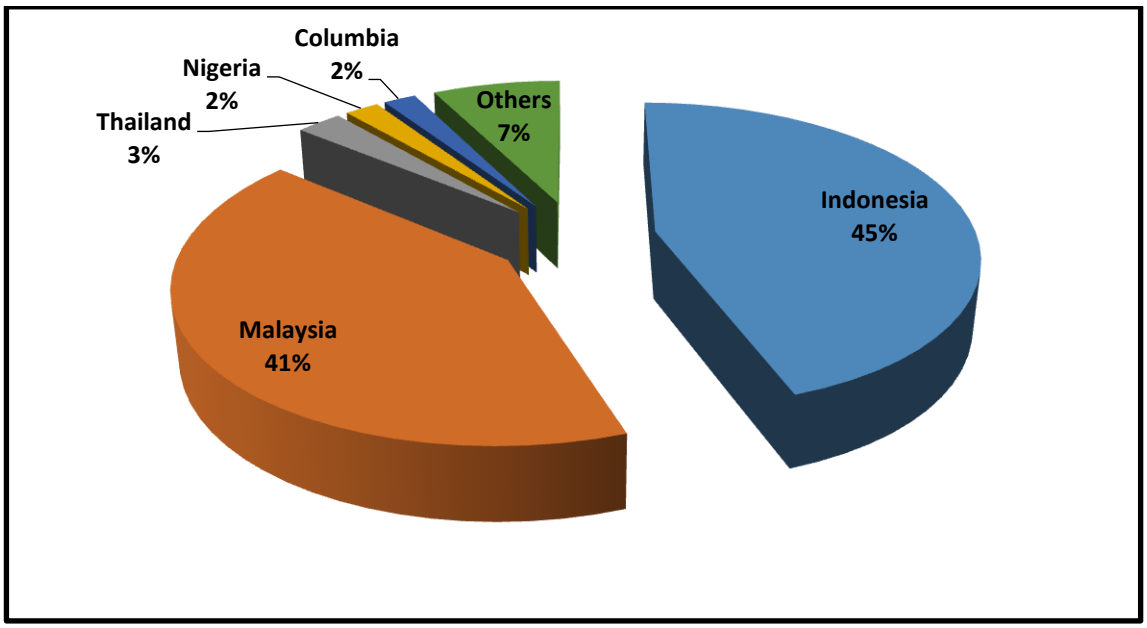

Figure 2.4: 2008 World palm oil productions' share (MPOB, 2008)

In Malaysia, the main product from oil palm plantation is palm oil and the secondary product is palm kernel oil and cake. The Malaysian Palm Oil Board (MPOB) long-term goals are to establish biodiesel plants and convert biomass from oil palm into value-added products. MPOB has a lot of research in the pipeline to make use of wastes generated from palm oil mill such as empty fruit bunch (EFB) and palm oil mill effluent.

2.2.2 Palm Oil Mill Effluent (POME)

Wastes from palm oil mill production are in solid or liquid form. Solid wastes mainly consist of palm kernel shells, mesocarp fruit fibres and empty fruit brunches. The wet process from the extraction of palm oil generates liquid wastes. This liquid waste combined with the waste from steriliser condensate and cooling water is called palm oil mill effluent (POME). Figure 2.5 shows the sources of waste in palm oil milling.

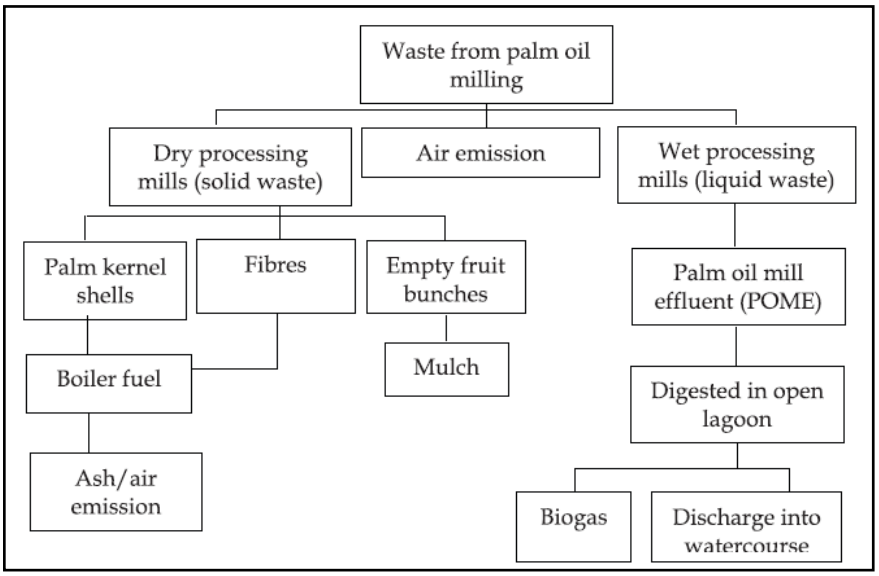

Figure 2.5: Sources of waste from palm oil milling (Ujang *et al*, 2012)

Fresh POME is thick, brownish, colloidal slurry of water, oil and fine cellulosic fruit residues. POME is generated from mill operation at temperature between 80°C and 90°C, with acidic pH between 4 to 5. The high organic matter is caused by the presence of sugars such as arabinose, xylose, glucose, galactose and mannose. The oil residue was very much dependent on quality of raw material, process control and machine efficiency. The suspended solids in the effluent are mostly oil-bearing cellulosic materials from the palm fruits.

POME poses environmental issues due to its large oxygen depleting capability if released in waterways due to its organic and nutrient contents, this can be seen in Table 2.2, where the BOD and COD is very high. However, POME is non-toxic since no chemical is added during the oil extraction process, thus it can be a good source of nutrients for microorganisms. Hence many studies have been done using POME as a substrate for microbes to produce value-added product. In the field of biohydrogen production, a study by Jamil, Mohamad Annuar, Ibrahim & Sabaratnam (2009) shown promising result when anaerobic co-digestion of POME with sewage sludge produced 1.05 ml hydrogen per ml of POME.

Table 2.2: Characteristics of palm oil mill effluent (POME) from palm oil mill (Ibrahim, 2012)

Parameter	Units	Concentration
Biochemical oxygen demand (BOD)	mg/l	10197
Chemical oxygen demand (COD)	mg/l	50500
Total solid (TS)	mg/l	31533
Suspended solids (SS)	mg/l	4007
Volatile suspended solids (VSS)	mg/l	3657
Oil and grease	mg/l	15800
Total nitrogen	mg/l	613
Dissolve oxygen (DO)	mg/l	0.47
Temperature	°C	54
pH	-	5.3

2.3 Biohydrogen production methods

Table 2.3 shows several methods for hydrogen production and their advantages and disadvantages. Among various hydrogen production processes, biological method is most ideal since it can utilise renewable energy sources such as biomass for the production of hydrogen and less energy-intensive. The biological methods mainly make use of photosynthetic hydrogen production and fermentative hydrogen production.

Major bioprocesses utilized for biological hydrogen production can be categorized into

- Biophotolysis of water by algae;

- Dark-fermentative hydrogen production (in acidogenic phase in anaerobic digestion);

- Two stage dark/photofermentative production of hydrogen.

Table 2.3: Comparison of various hydrogen production processes (Pandu and Joseph, 2012).

Process	Advantages	Disadvantages
Solar gasification	Good hydrogen (H_2) yield	Require effective solar collector plates
Thermo-chemical conversion	Higher conversion can be achieved	Need gas conditioning and tar removal
Pyrolysis	The process gives out carbonaceous material with oil, chemicals and minerals	Catalyst deactivation will occur
Supercritical conversion	Sewage sludge can be used easily, unlike in gasification	Selection of supercritical medium
Direct bio-photolysis	H_2 can be produced directly from water and sunlight	Requires high intensity of light, low photochemical efficiency and oxygen (O_2) is inhibitory
Indirect bio-photolysis	Blue green algae can produce hydrogen from water	Hydrogenates that algae uptake have to be removed
Photo-fermentation	A wide spectral of energy can be used by photosynthetic bacteria	O_2 is inhibitory on nitrogenase enzyme and efficiency of light conversion is low
Dark fermentation	It can produce hydrogen without light. No oxygen limitations and can produce several metabolites as by-products. Various substrates can be used in this process	Relatively lower H_2 yield. At higher H_2 yield, process becomes thermodynamically unfavourable
Two-stage fermentation	Can produce relatively higher H_2 yield. By-products can be efficiently converted to H_2	Requires continuous light source which is difficult for large scale processes

2.3.1 Biophotolysis of water by algae

In photosynthesis, water molecules are splitted to hydrogen ion and oxygen. The generated hydrogen ions are then converted into hydrogen gas by the enzyme hydrogenase. *Chlamydomonas reinhardtii* is one of the well-known hydrogen producing algae. There are two ways of hydrogen production with these method, either by direct or indirect biophotolysis. Equation (2.1) is for direct biophotolysis that utilizes the photosynthetic system of microalgae for the transformation of solar energy into the chemical energy needed for the splitting of water molecule to yield hydrogen.

$$2H_2O \xrightarrow{\text{solar energy}} 2H_2 + O_2 \qquad \text{(Equation 2.1)}$$

Hydrogenase activity has been detected in the green algae, *Scenedesmus obliquus,* in marine green algae *Chlorococcum littorale* and in *Chlorella fusca*. Cyanobacterial hydrogen gas evolution involves nitrogen fixing cultures such as non-marine *Anabaena* sp., marine cyanobacter *Oscillatoria* sp., *Calothrix* sp. and non-nitrogen fixing organisms such as *Synechococcus* sp., *Gloebacter* sp. and it was reported in literatures that *Anabaena* sp. have higher hydrogen evolution potential over the other cyanobacter species.

The algal hydrogen production could be considered as an economical and sustainable method in terms of wastewater utilization as a renewable resource and carbon dioxide consumption. The alga can be grown using nutrient-rich wastewaters for example from palm oil mill effluent (POME) or other non-toxic industrial wastewater. The system uptake carbon dioxide from the atmosphere which in turn reduces the pollutant level in the air thus reducing greenhouse gases effect.

However, strong inhibition effect of generated oxygen on hydrogenase enzyme is the major limitation for the process (Benemann, 2000). Inhibition of the hydrogenase enzyme by oxygen can be prevented by cultivation of algae under sulphur deprivation

for about 2 to 3 days; to provide anaerobic conditions in the light. Although biological processes for hydrogen production have been well documented with cultured microalgal biomass, these processes have to be integrated into a system to meet the overall efficiency of converting solar energy into fuels (Pandu and Joseph, 2012). Another disadvantage of hydrogen production by algae is low hydrogen production potential. Therefore, dark and photo-fermentations are considered the better options due to simultaneous waste treatment and biohydrogen gas production.

2.3.2 Dark-fermentation hydrogen production

Dark fermentation is conversion of organic substrate to hydrogen by fermentation. It is a complex process by a diverse group of bacteria through a series of biochemical reactions. Fermentative microorganisms hydrolyze complex organic polymers to monomers and then converting it to a mixture of lower molecular weight organic acids and alcohols. This is done by the hydrogen producing acidogenic bacteria.

Utilization of wastewater as a potential substrate for hydrogen production has been of interest in recent years; especially in dark fermentation process. Industrial wastewater as fermentative substrate for hydrogen production fulfil most of the criteria required for substrate selection *i.e* availability, low cost and biodegradability.

The use mixed culture is extremely important and suitable in wastewater treatment. This is due to the non-sterile, unstable and complex environment of wastewater. Some anaerobic mixed cultures only produce a small amount of hydrogen; as it is rapidly consumed by the methane-producing bacteria. Successful biological hydrogen production requires inhibition of these hydrogen-consuming microorganisms, such as methanogens by pre-treatment of the seed culture. This method is necessary for selecting the required microflora.

The physiological differences between hydrogen producing bacteria and hydrogen consuming bacteria are the fundamental basis to development of various pre-treatmnet methods. When the inoculum was exposed to extreme environments such as high temperature, extreme acidity and alkalinity, spore forming hydrogen producing bacteria such as *Clostridium* will survive. However methanogens had no such capability and will die off.

Many anaerobic organisms can produce hydrogen from carbohydrate-rich organic wastes. The organisms belonging to genus *Clostridium* are obligate anaerobes and spore forming organisms. *Clostrida* species produce hydrogen gas during the exponential growth phase. In batch growth of *Clostridia* the metabolism shifts from a hydrogen/acid production phase to a solvent production phase when the population reaches to the stationary growth phase. The dominant culture of *Clostridia* can be easily obtained by heat treatment of biological sludge. Im *et al* (2012) has done an analyses on bacterial community in dark fermentation by pyrosequencing. The study showed that at the start of fermentation, the microbes were very diverse with more than 10 phyla of bacteria, with 50% of it being *Proteobacteria.* However this decreased after 6 hours and members of the phylum *Firmicutes* were observed at more than 97%. The species in this phylum are *C. sordellii, C. perfringens* and *C. butyricum.*

Hydrogen production capacity of anaerobic facultative bacterial culture has been widely studied. Example of facultative anaerobes are *Enterobacter aerogenes*, *E. coli* and *Hafnia alvei.*

Environmental conditions are the major parameters to be controlled in hydrogen production. The pH of the medium affects hydrogen production yield, biogas content, type of the organic acids produced and the specific hydrogen production rate. Gradual decreases in pH inhibit hydrogen production since pH affects the activity of iron

containing hydrogenase enzyme. Therefore, pH control at the optimum level is required. Initial pH also influences the extent of lag phase in batch hydrogen production (Guo, Trably, Latrille, Carrere & Steyer, 2010). Composition of the substrate, media composition, temperature and the type of microbial culture are also important parameters affecting the duration of lag phase. Some studies reported that low initial pH of 4.0–4.5 causes longer lag periods while high initial pH levels such as 9.0 decrease lag time but then it lowers the yield of hydrogen production.

The major products in hydrogen production by anaerobic dark fermentation of carbohydrates are acetic acid, butyric acid and propionic acid. Formation of lactic acid was observed when lactose and molasses (sucrose) were used as the substrates. pH also affects the type of organic acids produced. More butyric acid is produced at pH 4.0–6.0. Concentration of acetate and butyrate could be almost equal at pH 6.5–7.0. Ethanol production was observed depending on the environmental conditions.

Methane was not detected in most of the hydrogen production studies since methane producers have been eliminated by pre-treatment. However, long retention times may still cause methane formation by the mesophilic cultures. Since the hydrogenase enzyme present in anaerobic organisms oxidizes reduced ferrodoxin to produce molecular hydrogen, external iron addition is needed for hydrogen production. Liu, Min & Angelidaki (2008) reported that high iron concentrations (100 mg /L) increases lag phase in batch operations. The composition of volatile fatty acids (VFA) may also vary as a result of metabolic shift in anaerobic digestion.

Nitrogen is an essential nutrient for hydrogen production by dark fermentation under anaerobic conditions. Some of hydrogen producers are strict anaerobes. Therefore, reducing agents gases such as argon, nitrogen and hydrogen are sparged into the bioreactor to remove trace amounts of oxygen present in the medium. However, the

use of such reducing agents gases are a bit costly, making it uneconomical for industrial-scale production of hydrogen gas.

Enterobacter aerogenes is a facultative anaerobe and the amount of hydrogen produced by this culture is comparable to *Clostridum* sp. The culture has the ability to survive in the presence of slight amount of oxygen generated during anaerobic biodegradation. Therefore, utilization of *E. aerogenes* along with *Clostridum* was done to eliminate use of reducing agents. This was proposed by Yokoi (2002) for effective hydrogen gas production by dark fermentation process.

2.3.3 Two stage dark/ photofermentative production of hydrogen

Sequential dark and photo-fermentation is rather a new approach in biological hydrogen gas production. There are not many literatures found on this sequential hydrogen gas production system. The sequential production system has certain advantages over single stage dark or photo-fermentation processes. The effluent of dark fermentation in hydrogen production provides sufficient amount of organic acids for the photo-fermentation. Hence there would not be any limitation of the organic acid availability. Further utilization of organic acids by photo-fermentative bacteria could provide better effluent quality with lower COD level.

However, the system should be well monitored and controlled to provide optimum environmental conditions for the two microbial components of the process. For instance, the ammonia concentration and C:N ratio in the effluent of anaerobic fermentation should not be at the inhibitory level for the photosynthetic bacteria. Dilution and neutralization of dark fermentation effluents are required before photo-fermentation to adjust the organic acid concentration and the pH to around pH 7 for the optimal performance of photosynthetic bacteria.

2.4 Microbiology of biohydrogen production from waste

Anaerobic digestion is a ubiquitous phenomenon found in anaerobic conditions. The first stages in anaerobic digestion are hydrolysis and acidogenesis, in which dark fermentation with hydrogen producers is involved. Afterwards, hydrogen as a key intermediate can be rapidly consumed by other microorganisms in mixed culture, mainly by homoacetogens, methanogens, and sulfate-reducing bacteria.

There are a lot of studies on the metabolic network of carbohydrate. Among the large range of end products generated by the various microbial metabolisms, acetic acid accumulates from acetic fermentation as the sole end product with a theoretical production of 4 mol H_2 /mol hexose (Equation 2.2). Meanwhile in the butyrate pathway, a lower molar hydrogen yield is observed with 2 mol H_2 /mol hexose (Equation 2.3).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \qquad \text{(Equation 2.2)}$$

$$C_6H_{12}O_6 \rightarrow \quad CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \qquad \text{(Equation 2.3)}$$

Keep in mind that the accumulation of acetate in the medium does not necessarily indicates a higher biohydrogen production since several microbial species can convert hydrogen and carbon dioxide to acetate.

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \qquad \text{(Equation 2.4)}$$

Normally in mixed cultures, ratio of 3:2 of butyrate/acetate is usually observed, resulting in a theoretical average hydrogen yield of 2.5mol H_2 /mol hexose (Hawkes *et al,* 2007). In mixed cultures, propionate, ethanol, and lactic acid may also accumulate. Propionate is a metabolite of a hydrogen-consuming pathway, while ethanol and lactic acid are involved in a zero-hydrogen balance pathway (Equations 2.5 – 2.7)

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \qquad \text{(Equation 2.5)}$$

$C_6H_{12}O_66 \rightarrow 2CH_3CH_2OH + 2CO_2$ (Equation 2.6)

$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH + 2CO_2$ (Equation 2.7)

Nandi and Sengupta (1998) has listed the major hydrogen-producing bacteria related to strict anaerobic genera (*Clostridia*, methylotrophs, rumen bacteria, methanogenic bacteria, archaea), to facultative anaerobic genera (*Escherichia coli, Enterobacter, Citrobacter*) and to aerobic genera (*Alcaligenes, Bacillus*).

Based on the biohydrogen production from agricultural waste, that is in mixed cultures, three classes of microorganisms could be identified; hydrogen producers, hydrogen consumers and metabolic competitors.

2.4.1 The biohydrogen producers

Eventhough pure cultures have been intensively studied in the past years, only a few studies refer to the characterization of mixed cultures. A large range of microbial sources has been used to obtain inocula for biohydrogen production. For example; anaerobic sludge from municipal wastewater plants and cow dung composts, cattle or dairy residue composts, palm oil mill effluent, soil, rice straw compost, fermented soy bean meal as well as landfill lixiviates.

From hydrogen-producing mixed cultures, a wide range of species have been isolated, more specifically from the genera *Clostridium* (*C. pasteurianum, C. saccharobutylicum, C. butyricum*), *Enterobacter* (*E. aerogenes*) and *Bacillus* under mesophilic conditions. Under thermophilic temperatures, there are microbes from the genera *Thermoanaerobacterium* (*T. thermosaccharolyticum*), *Caldicellulosiruptor* (*C. saccharolyticus*, *C. thermocellum*) and *Bacillus thermozeamaize*. Under mesophilic conditions, mainly sporulating bacteria of the *Clostridium* genus have been found in

mixed mixtures due to the use of heat shock and other pre-treatment method on the inoculums (Shin, Youn & Kim, 2004).

2.4.2 Hydrogen consumers and metabolic competitors

Three groups of bacteria are known to interfere, either directly or indirectly in hydrogen production from carbohydrates, such as the sulfate-reducing bacteria (SRB), the methane-producing bacteria (MPB) and the homoacetogenic bacteria (HAB). The pathway is shown in Figure 2.6.

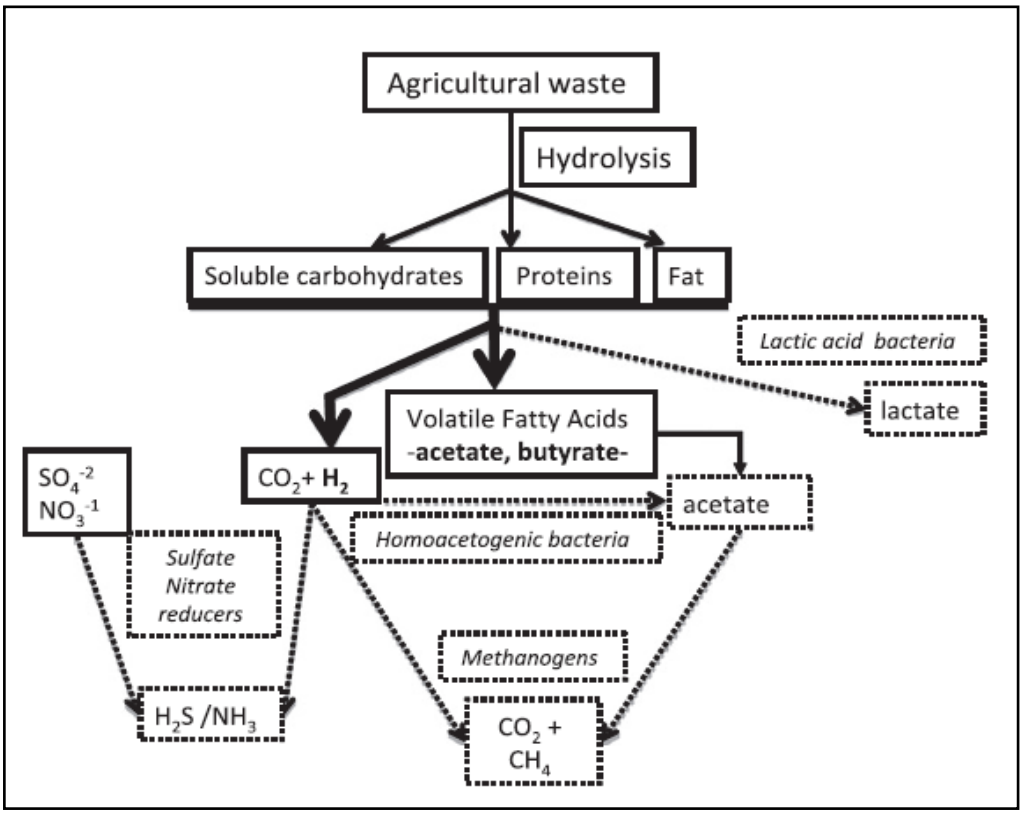

Figure 2.6: Microbial pathways in an ecosystem degrading agricultural waste. The bold arrows indicate hydrogen producing pathways, and dotted-line arrows indicate hydrogen-consuming pathways (Guo, Trably, Latrille, Carrere & Steyer, 2010)

2.4.2.1 Homoacetogenic bacteria

Homoacetogenic bacteria are strictly anaerobic microorganisms which catalyze the formation of acetate from hydrogen and carbon dixide. They were first observed by Fischer *et al.* (1932). *Clostridium aceticum* and *Clostridium thermoaceticum* were the

model species used to show the metabolic pathway. They have special enzymes that can catalyze the formation of acetyl-CoA, which is later converted either to acetate in catabolism, or to cell carbon in anabolism (Guo, Trably, Latrille, Carrere & Steyer, 2010).

The homoacetogens are versatile; they can convert a variety of different substrates to acetate as the major end product. This means that the biohydrogen production obtained may be lower than the expected value due to the accumulation of acetate (Antonopoulou *et al*, 2008). It was shown that the biohydrogen produced from butyrate oxidation reacted rapidly with carbon dioxide to form acetate by homoacetogenesis.

Unfortunately, the pretreatment of the inoculum by heating; method to select spore-forming bacteria, will not successfully inhibit homoacetogenic bacteria since some of them belong to the same genus *Clostridium*.

2.4.2.2 Sulfate-reducing bacteria

According to theoretical thermodynamics, the most efficient biochemical reaction using hydrogen involves the sulphate and nitrate-reducing microorganisms. It has been shown that SRB have a thermodynamic advantage over MPB and HAB (Valdez *et al,* 2009). Some waste especially from pulp and paper industry, sea-food processing, distilleries, edible oil and wet corn milling, contains high concentrations of sulfate which will disturb anaerobic digestion. It also produces sulfide gas which is hazardous for fuel cells.

Short hydraulic retention times (HRT) are not sufficient to inhibit these microorganisms. At longer HRT, hydrogen is converted either to methane or sulphuric acid. Under sulphate-limited conditions, methane and carbon dioxide are produced by the MPBs from hydrogen. In the event of abundance of sulphate, the SRB will convert

hydrogen to sulfidic acid. Along with the concentration of sulfate and HRT, pH is also a key factor in sulfate reduction. At pH values lower than 6 the activity of SRB is inhibited (Mizuno *et al*, 1998).

2.4.2.3 Methanogens

Methanogens are considered as the main hydrogen consuming microorganisms in anaerobic environments. Many options exist to inhibit methanogenesis: chemical inhibition, low pH control, heat treatment of the inoculums and short hydraulic retention times.

The most commonly used chemical inhibitors are bromoethanesulfonate (BES), acetylene and chloroform BES. These are specific against methanogens; it acts as an analog of the coenzyme M in the respiratory chain. However, BES is not environmentally friendly and it is too costly for industrial scale (Li and Fang, 2007).

Most methanogens can only grow at a narrow pH range from 6 to 8. In absence of pH control during a batch process, an acidic initial pH is strongly recommended (Chen, Chen, Khanal & Sung, 2006). The most common treatment of inoculum is by heating to around 100°C to select spore-forming, hydrogen-producing bacteria. Methanogens do not sporulate, hence will not survive such thermophilic conditions. A short HRT of less than 8 hours will lead to a washout of methanogens from the reactor, provided no biofilm is formed. To obtain stable hydrogen production in a methane-free biogas, the optimal HRT observed were 48 hours for food waste (Shin, Youn & Kim, 2004).

2.4.2.4 Lactic acid bacteria

Lactic acid bacteria (LAB) growth could not be limited by temperature. The accumulation of lactic acid led to the instability of the mixed culture processes. Indeed,

Wang & Wan (2009) showed that lactic acid inhibited hydrogen fermentation in a two stage continuous system using food waste as substrate. The hydrogen yield dropped from 71 to 49 ml hydrogen /g volatile solid (VS) when the lactic acid increased from 2.3 to 4.4 g/L.

2.5 Types of Waste Materials for Biohydrogen Production

The main things to look for in selecting waste materials to be used in biohydrogen production are abundance, cost, carbohydrate content and biodegradability of it. Simple sugars, for example glucose, sucrose and lactose are readily degradable and are the preferred substrates for hydrogen production. However, the cost to obtain a pure carbohydrate sources are very high, thus making it less economical for a production system.

2.5.1 Food industry and agricultural waste

Food waste is a major solid waste in the world; it is problematic and abundant everywhere. It is the major source of bad odour, vermin attraction, toxic gas emission and groundwater contamination. Food waste and also waste from food industries constitute a big fraction of the municipal food waste. Conventional approaches for solid waste management, such as landfilling, composting and incineration for these wastes. Seeing that these wastes have high content of carbohydrate in the form of simple sugars, such as starch and cellulose, made it a potential feedstock for hydrogen production.

Production of clean and green energy source and utilization for minimization of waste materials make hydrogen production from food waste a new and promising approach to meet the increasing energy demands and a substitute for fossil fuels.

There are extensive studies on utilizing food waste as carbon source for fermentative hydrogen production because it is rich in carbohydrate and also easily hydrolysable in water. But the problem with the food waste is the variations in carbohydrate and protein types, and their concentrations in the mixture. Each component requires different environmental conditions for hydrogen gas production. Table 2.4 below summarizes hydrogen gas production from a few types of wastewaters and solid wastes.

Table 2.4: Yields and rates of biohydrogen production from different waste materials by dark fermentation (Kapdan and Kargi, 2006).

Organism	Carbon Source	SHPR	$Y_{P/S}$ yield coefficient	Percentage of hydrogen content (%)	References
Mesophilic mixed culture	Food waste (3% VS)	0.7ml/g VSS h	0.05 mol/mol hexose	1	Shin *et al,* 2004
Mixed culture	Food waste (3% VS)	111ml/g VSS h			Kim *et al,* 2004
Mixed culture	Potato industry WW (21g COD/L)			2.8 L/L WW	Ginkel *et al,* 2001
Mixed culture	Rice winery	389ml/g VSS h	2.14 mol/mol hexose	53-61	Yu *et al,* 2002
C. butyricum and *E. aerogenes*	Sweet potato starch residue (2%)			2.7mol/mol glucose	Yokoi *et al,* 2002

SHPR, Specific Hydrogen Production Rate; WW, Wastewater.

2.5.2 Palm oil mill effluent (POME)

Palm oil is a major crop in tropical countries such as Malaysia itself. During extraction of crude palm oil, palm oil mill effluent (POME) will be generated. The BOD

and COD of POME are way too high to be discharged into waterways. Pre-treatments need to be done to reduce the harmful effect of POME when released in domestic water. Conventionally POME is treated using pond system or open digestion tank. The problems with these methods are that it has long hydraulic retention time (HRT), gives out terrible odor and also the containment and collection of gas produced is troublesome.

In recent years, more studies have been done to find other alternatives to value-add POME. POME has been used as a substrate to produce compost and citric acid production. POME has also been used to produce hydrogen and based on studies done the hydrogen production is comparable to those produced using carbohydrate-rich wastewater (Chong, Sabaratnam, Shirai & Hassan 2009). Most of these are still at laboratory scale but all are showing promising results.

2.5.3 Dairy wastewater

Lactose rich wastewater from dairy and cheese industry also contains complex organics such as polysaccharides, proteins, lipid which can form sugars, amino acids and fatty acids through hydrolysis. These intermediate products are converted into volatile fatty acids (VFA) and can be further degraded by acetogens to form acetate, carbon dioxide and hydrogen (Mohan *et al*, 2007). Cheese whey contains about 5% lactose which makes it eligible to be a substrate for fermentation process (Chong, Sabaratnam, Shirai & Hassan 2009).

A study by Moreno-Davila, Rios-Gonzalez, Garza-Gracia, Garza & Rodriguez-Martinez (2011) studied the fermentative hydrogen production in packed bed batch reactors to assess the influence of environmental factors such as initial COD, temperature and pH. Table 2.5 shows the dairy wastewater composition from this study. The maximum yield obtained was 12.73 mM hydrogen per gram COD.

Table 2.5: Dairy wastewater composition (Moreno-Davila, Rios-Gonzalez, Garza-Gracia, Garza & Rodriguez-Martinez, 2011)

Parameters	Dairy Wastewater
pH	11.32 ± 0.240
COD	21.1 ± 0.381
Conductivity	2640 ± 52.8
TSS	21.9 ± 0.557

2.5.4 Lignocellulosic materials

Lignocellulosic materials, such as cellulose, hemicelluloses and lignin, form the structural component of a plant cell wall. They are available in bulk as waste from the wood and agricultural industries. These materials are the largest renewable sources of hexose and pentose sugars for industrial fermentation and have good potential to be resource for biohydrogen production too. However, the need for a pretreatment process to degrade these polymers into simple sugar has limited its use.

Utilization of cellulose degrading bacteria is a great alternative to chemical pretreatment. For efficient hydrolysis of cellulosic materials, the bacterial cell has to adhere to the cellulose. A study by Levin, Islam, Cicek & Sparling (2006) utilizes *Clostridium thermocellum* for biohydrogen production using cellulose-based medium, reported that delignified wood fibers were good substrates and produces yield of 1.6 mol hydrogen per mol glucose.

2.6 Biological Reactor Operation

At industrial scale, the main concerns are the low productivity and the low conversion yields of the fermentative biological processes. Based on the current hydrogen

productivity, industrial processes would require very large-volume reactors. The productivity of hydrogen-producing bioreactors treating agricultural waste and food waste is quite low. This is due to the use of complex and polymeric organic substrates, and also the use of mixed cultures as inoculum.

The optimization of the operating conditions in biological reactors is the key for the improvement of biohydrogen production. Specifically-optimized bioreactors could help to determine whether the use of food waste would be feasible technically and economically. In order to meet these requirements, some operating conditions must be considered such as pH, temperature and hydrogen partial pressure.

2.6.1 pH

pH is one of the most important factors to be controlled in anaerobic digestion system. pH can affect the hydrogen yield in mixed cultures and can also modify the by-product which in turn affects the structure of microbial communities.

Different substrate has different optimal pH for hydrogen production. For example, optimal hydrogen production appears to take place with a pH of 5.0 to 6.0 for food wastes whereas a neutral pH is suitable for crop residues and animal manure. Li *et al* (2007) investigated a large range of initial pHs, from 4 to 8, in batch tests. The results showed that pH of 7.0 to 7.5 as optimal for the conversion of corn straw to biohydrogen. The accumulation of acidic byproducts, such as acetate and butyrate, will lower the pH of the medium.

It was concluded that a pH of 5.5 is optimal for hydrogen production. It can be said that in general, the optimal pH in terms of biohydrogen production is within a range of 5.0 to 7.0. This pH range probably favors the activity of the hydrogenases and is suitable for microbial development in dark fermentation (Li and Fang, 2007). The pattern of intermediate VFAs is different under various pH conditions. Butyrate and

acetate are the two main by-products of anaerobic digestion, but at low pHs butyrate is preferentially produced. Hydrogen-producing butyrate-acetate pathways are favored at pH 4.5 to 6.0 while at neutral or higher pH conditions, ethanol and propionate will accumulate.

It should be taken into consideration that under both high and low pH conditions, the fermentation pattern was clearly associated with the dominance of *Clostridium* species. At intermediate pHs however, metabolic shifts involved higher microbial diversity.

2.6.2 Temperature

Temperature is one of the most important parameters affecting both the biohydrogen production and microbial metabolisms in mixed cultures. Due to the complexity of the food waste and operating conditions, no optimal temperature for hydrogen fermentation can be concluded from the data in literatures.

Most studies on fermentative hydrogen production have been based on mesophilic temperatures. Li *et al* (2009) reported that out of 101 case studies, 73 were carried out at mesophilic temperatures. As for food waste, thermophilic temperatures seem more suitable for hydrogen production although some may disagree. These differences probably due to the various type of inoculum used, the different amount of readily-biodegradable compounds as well as the operating conditions. At 55°C, acetate was the dominant by-product while a propionate production pathway was favored at 20°C (Karlsson, 2008).

Without pretreatment of the initial inoculum, temperatures higher than 60°C are recommended in order to reduce hydrogen-consuming activity. However, the main disadvantage of thermophilic anaerobic fermentation processes is the energy requirement for heating and maintenance.

2.6.3 Hydrogen partial pressure

A few studies reported that partial pressure of hydrogen is a restrictive factor in the fermentation of organic waste. The oxidation of reduced components to VFAs, alongside hydrogen production, will results in a low biohydrogen concentration in the medium. This is because reactions are thermodynamically unfavorable. The positive Gibbs energy of long chain fatty acid (LCFA) degradation shows that the degradation of fat through the β-oxidation pathway is thermodynamically unfavorable and therefore it requires an extremely low level of hydrogen partial pressure (Equation 2.8) (Li, 2009).

$$\text{n-LCFA} \rightarrow \text{(n-2)-LCFA} + 2\text{Acetate} + 2H_2 \quad \Delta G = +48\ \text{kJmol}^{-1} \text{ (Equation 2.8)}$$

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + CO_2 \quad \Delta G = +104.6\ \text{kJ mol}^{-1} \text{ (Equation 2.9)}$$

Additional hydrogen could also be derived from the degradation of acetate (Equation 2.9). This conversion is thermodynamically unfavorable at mesophilic temperatures. The reaction is therefore extremely sensitive to hydrogen concentration. The inverse reaction is called homoacetogenesis. It is favored in the fermentation process and plays a part in reducing the performance of bioreactors through the accumulation of acetate in the medium.

When the hydrogen concentration in the medium increases, not only biohydrogen production may be affected but also a shift of metabolic pathways towards solventogenesis has been observed such as the accumulation of lactate, ethanol, acetone and butanol (Levin, Islam, Cicek & Sparling, 2006).

Agitation is most common method to decrease the hydrogen partial pressure in the medium, especially in highly concentrated bioprocesses treating organic waste, as reported by Chou (2003). Mizuno *et al* (1998) showed that sparging nitrogen gas into a fermentor fed with simple sugars led to double the biohydrogen yield from 86.76 ml

H_2/g VS to 187.86 mL H_2/g VS. The main disadvantage of sparging techniques is that, regardless of the significant pressure removal, the sparged gas dilutes the biohydrogen content and creates a further reduction in separation efficiency. Also, the sparging processes are highly costly on industrial scale and hydrogen purification would raise the production costs.

Membrane absorption techniques offer an energy-effective alternative for hydrogen removal. Despite the different techniques available for reducing the partial hydrogen pressure, more research is still required to develop efficient and low cost gas purification system.

2.7 Waste to Wealth: Potential of using wastes such as POME, food waste and sewage sludge for biohydrogen production in Malaysia

Malaysia is steadily progressing towards green energy technologies. Figure 2.7 shows the overview of renewable energy sectors. The Malaysian government has made a few policies and programmes to encourage development of green energy. The Green Technology Policy was established in 2009 comprising of four main areas: Energy, Environment, Economy and Social. Several incentives and special yearly budgets are also devised for this purpose. Budget 2001 was the first time special treatment was given to renewable energy and in Budget 2010, incentive was offered to companies that comply with GBI.

A clean development mechanism (CDM) was developed based on two treaties; UNFCCC Rio de Janeiro Earth Summit 1992 and Kyoto Protocol 1997. The Kyoto protocol calls for greenhouse gases (GHG) reduction during 2008 to 2012. CDM allows trading of certified emission reductions (CER) between developing and Annex I

countries. 1 CER is equivalent to 1 tonne of carbon dioxide (Dagoumas, Papagiannis & Dokopoulos, 2006).

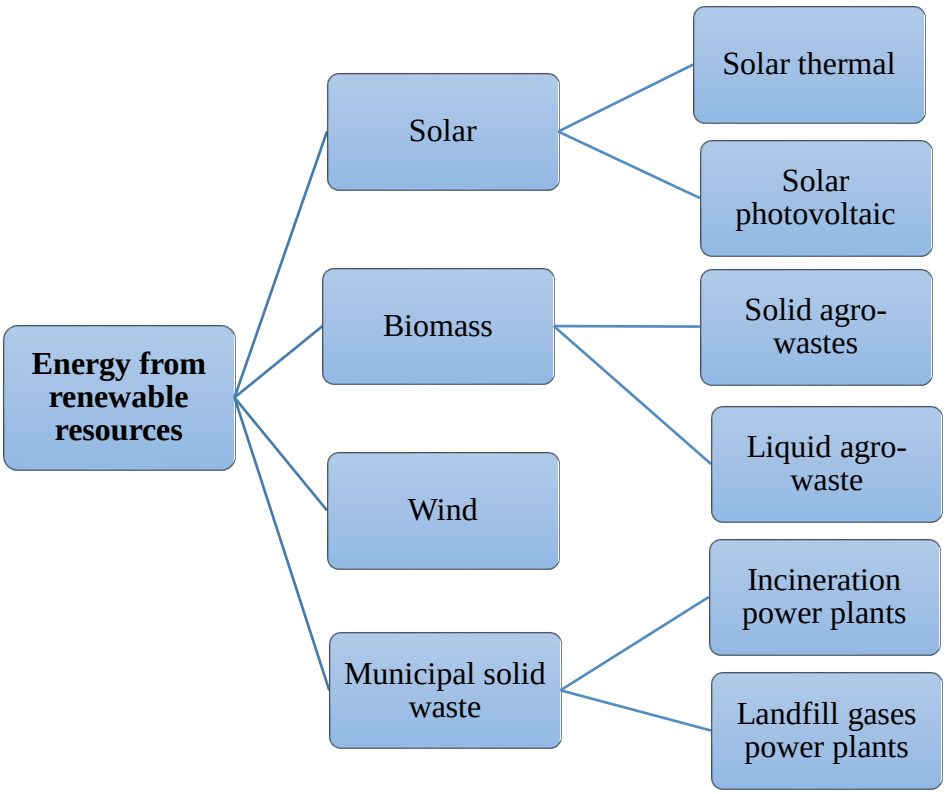

Figure 2.7: Overview of renewable energy sectors and green technology development.

Promoting renewable energy has been such a hassle throughout the world due to the higher capital cost to develop and run it when compared to the conventional goal and natural gas power. It was reported that Malaysia emits 187 million tonnes of carbon dioxide per annum, which is 7.2 tonnes per capita. This is already exceeding the world average of 4.3 tonnes per capita. Malaysia has pledged to reduce the emission 40% by the year 2020 and that the annual energy consumption growth would reduce while still maintaining gross domestic product (GDP) growth.

Five projects from Malaysia has been awarded and claiming CER worth total of RM 12.3 million. The first Malaysian project to be registered is Biomass Energy Plant in Lumut, Perak of the ENCO Energy and PGEO Energy companies. The Lafarge

cement has the first large scale CDM project in Malaysia to be registered. The plant replaced coal with palm kernel shell to generate power (Economic Planning Unit, 2009)

Table 2.6: Compare and contrast of different power options with the carbon dioxide emitted and their costs. (Economic Planning Unit, 2009)

Power options	CO_2 emitted (grams/ kilowatt/ hour)	Cost (US cents/ kilowatt/ hour)
Coal	947	5.1
Natural gas	159	3.8
Solar	121	59.8
Small scale hydro	40	8.9
Nuclear	32	6.8
Wind	24	10.5

Municipal solid wastes (MSW) and palm oil mill solid wastes and effluents have big potential to become a resource for renewable energy. MSW can produce energy by two technologies; incineration and landfill gas such as methane. However there are still some transactional economic barriers. On the other hand, for POME a few other methods can be utilized for energy conversion; biomass energy through combustion or gasification of empty fruit bunch (EFB), biogas (methane) from POME and bioethanol from EFB by fermentation method.

2.8 RSM application for biohydrogen production

Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes. The most common applications of RSM are in situations where several input variables potentially

influence some performance measure or quality characteristic of the process. So performance measure or quality characteristic is called the response. The input variables are sometimes called independent variables, and subjected to the control of the experimenter. The field of RSM consists of the experimental strategy for exploring the space of the process or independent variables, empirical statistical modeling to develop an appropriate approximating relationship between the yield and the process variables, and optimization methods for finding the values of the process variables that produce desirable values of the response.

In this work, RSM using a full factorial design will be used to determine the optimal conditions of temperature, inoculums size and pH for biohydrogen production from food waste using a mixed culture from pre-treated sludge.

3.0 MATERIALS AND METHODS

3.1 Reagents

All the reagents were prepared in accordance to the Standard Methods 18th edition (APHA, 1992)

3.1.1 COD Analysis (APHA,1992)

- Potassium Hydrogen Phtalate (KHP) solution for standard curve
- Potassium dichromate ($K_2Cr_2O_7$) digestion solution 0.017 M
- Sulphuric acid reagent

3.1.2 Total Kjeldahl Nitrogen (TKN) Analysis (APHA,1992)

- TKN digestion reagent
- Sodium Hydroxide- Sodium Thiosulfate reagent
- Indicating boric acid solution
- Mixed indicator solution
- Sulphuric acid titrant 0.02 N

3.2 Sample Collection and Preparation

3.2.1 Preparation of substrate

Food waste was collected from various cafeterias around University of Malaya. It consisted mainly of rice, vegetables and chicken. After removing the bones and unnecessary waste, the food waste was grounded using an electrical blender. The food waste was mixed with tap water to facilitate blending. After blending, the food waste is filtered using domestic sieve with pore size 0.2 mm to remove excess water. The blended waste was then packed into small plastic bags of 1 kg each and then kept in

freezer at -20°C. Before being used in experiment, the food waste was thawed for overnight.

The chemical characteristics of food waste were analyzed. The pH was around 4.0 to 4.3, with total suspended solid (TSS) and volatile suspended solid (VSS) of 762.7 mg/l and 526.7 mg/l, respectively.

3.2.2 Sewage Sludge

The bacterial population used as inoculum in the production of H_2 was from anaerobic sludge obtained from Indah Water Konsurtium from the Pantai Dalam treatment plant. The sludge was obtained from the gravity thickener part of the sludge holding tank, before it enters the anaerobic digester.

The sludge is kept in cold room at 4°C and thawed before use. The sludge will be heat treated prior use to kill off methanogens in a water bath at 100°C for an hour (Mohammadi, Ibrahim, Mohamad Annuar & Law, 2011; Ginkel and Sung, 2001). The pH of the sludge was 7.25, with total suspended solid (TSS) and volatile suspended solid (VSS) of 284 mg/l and 244 mg/l, respectively.

3.2.3 Palm Oil Mill Effluent

The palm oil mill effluent (POME) was collected from a local palm oil in Selangor. The collected POME was stored in cold room at 4^0C. This is to minimize and slow down the degradation of the effluent from microbial action. The characteristics of the POME were analyzed. The pH is 4.2 to 4.55, with total suspended solid (TSS) and volatile suspended solid (VSS) of 833.2 mg /l and 567.5 mg /l, respectively.

3.3 Experimental Design and Procedure

3.3.1 Inoculum Preparation

For inoculum preparation, the pre-heated sludge was cultivated in food waste and incubated in a shaker for 24 hours (ratio of sludge to food waste is 1:4) before being used as the inoculum in the experiment. The temperature and initial pH used were according to the intended combination to be tested in the experimental design.

3.3.2 Comparison between Anaerobic and Facultative Anaerobic Bacteria for Hydrogen Production

Anaerobic condition is established by sparging the serum bottles with oxygen-free nitrogen gas for 3 minutes at 20 ml/ minute. Facultative anaerobic condition for the experiment was obtained by sealing the serum bottles without any nitrogen gas sparging. The parameters chosen are listed in Table 3.1 for both aerobic and anaerobic conditions. The experiments were done in triplicates and the resulting cumulative hydrogen production obtained was averaged.

Table 3.1: Experimental parameters for both anaerobic and facultative anaerobic conditions

Parameters	Levels			Conditions
Initial pH	4.5	5.5	6.5	35°C, inoculum size 20%
Temperature (°C)	35	45	55	Initial pH 4.5, inoculum size 20%
Inoculum size (% volume/ volume) (ml)	2	11	20	Initial pH 4.5, 35°C

3.3.3 Full Factorial Design

Full factorial design allow for simultaneous study of several factors and their interactions' effects on resulting production. Varying the levels of the factors simultaneously rather than one at a time is time and cost efficient.

The high and low levels defined for the full factorial design are shown in Table 3.2. The high and low levels were chosen based on literature review. The response (cumulative hydrogen production and COD removal) was determined as an average of three simultaneous experiments.

For each parameter studied, three points were set up; a minimum, middle and maximum point *viz* temperatures of 35°C, 45°C and 55°C; inoculum sizes of 2%, 10% and 20% volume/volume (ml); initial pH of 4.5, 5.5 and 6.5. Using Minitab Pro 16.1, a full factorial design for RSM was developed as shown in Table 3.3. Standard order shows what the order of the runs in the experiment would be if the experiment was done in standard order. Run order shows what the order of the runs in the experiment would be if the experiment was run in random order.

Table 3.2: Variables and levels used in the factorial design

Symbols	Variables	Levels		
A	Initial pH	4.5	5.5	6.5
B	Temperature (°C)	35	45	55
C	Inoculum size (% volume/ volume) (ml)	2	10	20

Table 3.3 is the RSM design by Minitab software. Each experimental condition is a run. Run order is the randomized standard order in which each set of test conditions is run. Randomization is the best practical technique to prevent confounding between time and factor of interest though it doesn't give guarantee.

Table 3.3: RSM Design by Minitab Pro 16.1.0.0 Software

Standard Order	Run Order	Center Point	Blocks	pH	Temperature	Inoculum size
3	1	1	1	4.5	55	2
24	2	1	1	6.5	55	20
5	3	1	1	4.5	35	20
4	4	1	1	6.5	55	2
1	5	1	1	4.5	35	2
26	6	0	1	5.5	45	11
20	7	1	1	6.5	55	2
11	8	1	1	4.5	55	2
9	9	1	1	4.5	35	2
12	10	1	1	6.5	55	2
7	11	1	1	4.5	55	20
17	12	1	1	4.5	35	2
21	13	1	1	4.5	35	20
19	14	1	1	4.5	55	2
25	15	0	1	5.5	45	11
22	16	1	1	6.5	35	20
6	17	1	1	6.5	35	20
14	18	1	1	6.5	35	20
10	19	1	1	6.5	35	2
23	20	1	1	4.5	55	20
27	21	0	1	5.5	45	11
15	22	1	1	4.5	55	20
16	23	1	1	6.5	55	20
2	24	1	1	6.5	35	2
13	25	1	1	4.5	35	20
18	26	1	1	6.5	35	2
8	27	1	1	6.5	55	20

3.3.4 Experimental Design

The batch experiment was conducted in 120 ml serum bottles with a working volume of 50 ml, with varying initial pH, inoculum size and temperature. Figure 3.1 shows the experimental design of this study. There were 2 designs; Production 1, with food waste as inoculum and Production 2, with food waste and POME as inoculum. The mixing ratio for food waste and POME in Production 2 was 50:50 (v/v).

A control batch is also run with solely food waste without the sludge as inoculum. The bottles were sealed with rubber stopper and aluminium cap. The pH is controlled using 1M NaOH and 1M H_2SO_4. The serum bottles were sparged with oxygen-free nitrogen gas for 3 minutes at 20 ml/minute to provide a fully anaerobic condition.

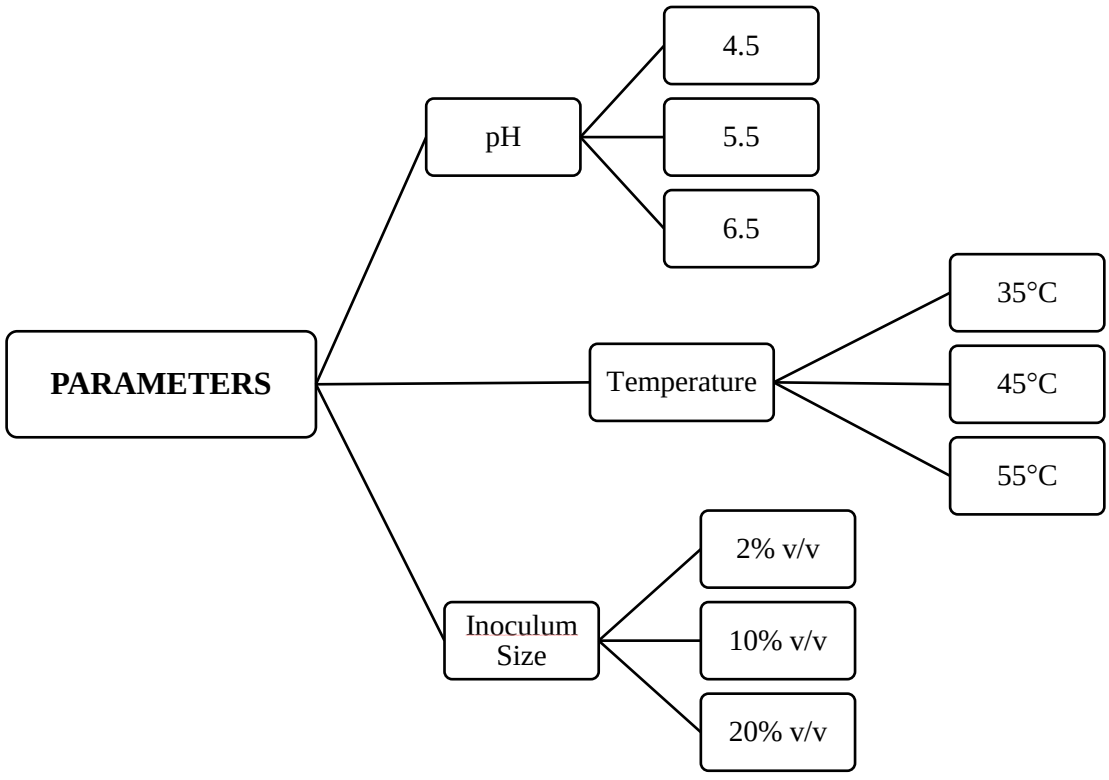

Figure 3.1: Experimental design to study hydrogen production from food waste and mixed culture (Production 1) food waste with POME and mixed culture (Production 2)

All experiment was conducted in an incubator shaker at 150 rpm for 72 hours. Figure 3.2 shows the incubator shaker used for this experiment. Biogas sampling was done at every 8 hours interval. The biogas collected using syringes are kept in acidic water (around pH 2) in 20 ml serum bottles using water displacement method, as shown in Figure 3.3. The acidic pH helps to prevent the gas from dissolving into the water. The serum bottles were then covered with a rubber septa and aluminium caps as shown in Figure 3.4.

The gas content was analyzed using a gas chromatography machine with thermal conductivity detector (TCD). All treatments were done in 3 replicates.

Figure 3.2: The experiments were conducted in an incubator shaker for 72 hours

Figure 3.3: Biogas produced was stored in acidic water (pH 2) using water displacement method

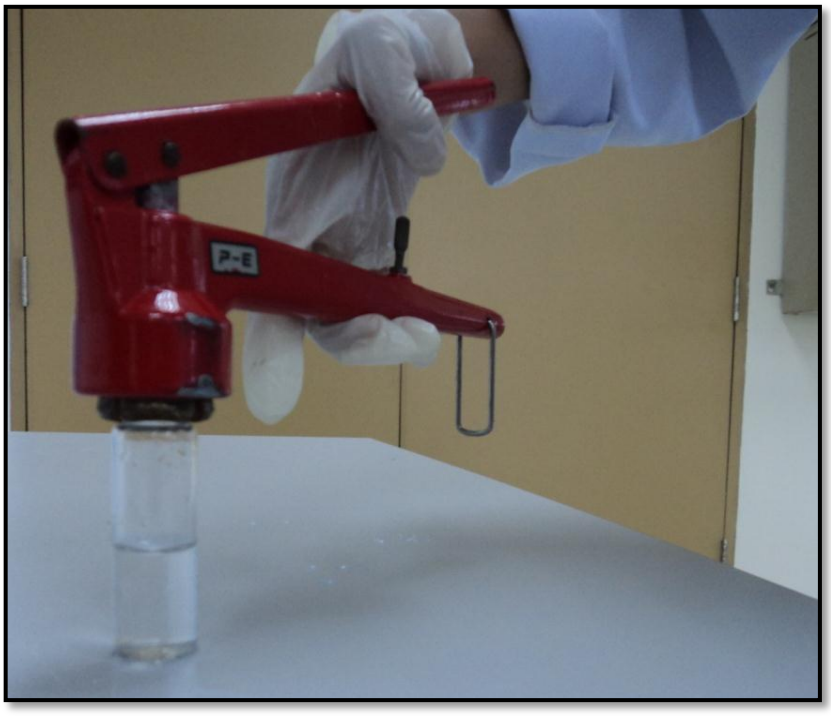

Figure 3.4: The serum bottle was sealed with a rubber septa and aluminum cap

3.4 Analytical methods

3.4.1 Hydrogen Analysis

The biogas composition was determined using a gas chromatograph (Perkin Elmer, AutoSystem GC) equipped with thermal conductivity detectors (TCD) and digital data acquisition system as shown in Figure 3.5. Hydrogen content was analyzed by GC-TCD fitted with a 1.5m stainless steel column packed with a molecular sieve (80/100 mesh) the temperatures of injection port, oven and detector were 80^0C, 200^0C and 200^0C respectively. The carrier gas used was Argon at a flow rate of 30 ml/min. A volume of 1 ml of gas sample was injected in triplicates.

Figure 3.5: Gas chromatography machine (Perkin Elmer Autosystem GC)

Hydrogen gas production was calculated from headspace measurement of gas composition and the total volume of hydrogen produced using the mass balance equation (Jamil, Mohamad Annuar, Ibrahim & Sabaratnam, 2009):

$$V_{Hi} = V_{Hi-1} + C_{Hi}(V_{Gi} - V_{Gi-1}) + V_{H0}(C_{Hi} - C_{Hi-1}) \quad \text{(Equation 3.1)}$$

Where:

V_{Hi} is the cumulative hydrogen gas volumes at the current (i), V_{Hi-1} is the previous time interval ($_{i-1}$)

V_{Gi} is the total biogas volume at the current time interval, V_{Gi-1} is the total bioghas volume at previous time interval

C_{Hi} is the fraction of hydrogen gas in the headspace at the current time interval

V_H is the volume of headspace of the serum bottle (70ml)

3.4.3 TSS and VSS analysis

Total suspended solids are materials that were retained on a standard glass fiber filter paper when a sample of wastewater is filtered. The residue on the filter paper was dried overnight in the oven at 105°C. The glass fiber filter paper used in this study was Whatman filter paper, sized 47 mm with pore size of 0.75 µm.

The TSS and VSS analysis was done according to the standard method. For TSS analysis, the filter paper was first dried in an oven at 105°C and cooled in dessicator. The filter paper was then weighted. The filter paper was placed on the filtration apparatus and filtered under vacuum condition. 50 ml of well-mixed sample is filtered until all the water was removed. The filter paper was then transferred into a porcelain

crucible and dried in an oven overnight. The filter papers were then cooled in a dessicator and weighed. These filter papers are then dried at 550°C for 15 minutes for VSS analysis. The papers are dried in dessicator and then weighed.

Calculation for TSS and VSS analysis is shown in Equation 3.2:

Total Suspended Solids = [(A – B) / C] X 1000mg/L X Dilution factor (Equation 3.2)

where:

A = Weight of filter paper with residue (mg)

B = Weight of filter paper (mg)

C = ml of sample taken

3.4.4 Chemical Oxygen Demand Analysis

The chemical oxygen demand (COD) determination is a measure of the oxygen equivalent of that portion of the organic matter in a sample that is susceptible to oxidation by a strong chemical oxidant. The COD analysis is done using the closed reflux colorimetric method.

A sample volume of 2.5 ml was measured into COD tube cell. Then, 1.5 ml of potassium dichromate solution was added. After that, 3 ml of sulphuric acid reagent was added slowly into the cells. All cells were then heated at 150ºC in heating blocks for two hours. The cells were subsequently cooled down to room temperature. The samples' absorbance values were read using a spectrophotometer at 600nm. The absorbance value was read in triplicates, and deducted by the value of blank. The absorbance value of the sample was then compared to the standard calibration (Figure 3.6) to determine the oxygen concentration as mg oxygen /liter.

A blank was prepared in a similar manner with the sample replaced by distilled water. A standard calibration was prepared using potassium hydrogen phtalate solution, using the same procedure as before, with varying concentrations from 0 to 500 mg/l.

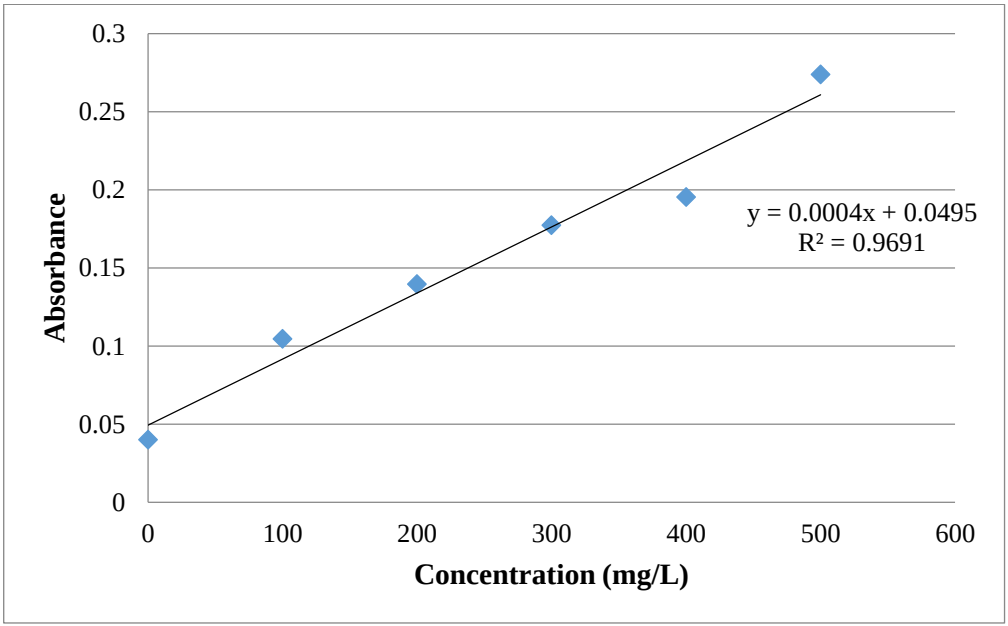

Figure 3.6: Standard calibration curve for COD analysis

3.4.5 Total Kjeldahl Nitrogen (TKN) Analysis

Nitrogen exists in water in the form of nitrate, nitrite, ammonia and organic nitrogen. All these forms of nitrogen, as well as nitrogen gas (N_2) are biochemically inconvertible and are components of the nitrogen cycle. Nitrate is an essential nutrient for many photosynthetic autotrophs.

Kjeldahl nitrogen is the sum of organic nitrogen and ammonical nitrogen. The macro-kjeldahl method used in this study is applicable for samples containing either low or high concentrations of organic nitrogen, but requires a large sample volume for low concentrations. Samples for TKN can be stored by acidifying it to pH 2 with concentrated sulphuric acid and storing them at 4°C.

3.4.5.1 Digestion

A volume of 25 ml of digestion reagent and 25 ml of sample was added into the distillation flask. Two glass beads are added to facilitate mixing. A blank was prepared by replacing the sample with distilled water. The flasks were boiled slowly at 165ºC for about 2 hours.

The flasks were left to cool down to room temperature before diluting it to 150 ml with distilled water and mixed. The flasks were tilted carefully and 25 ml of sodium hydroxide thiosulfate reagent was added to form an alkaline layer at the bottom.

3.4.5.2 Distillation

The flask was connected to a distillation apparatus and the flask were swirled to ensure complete mixing. After distillatiom, 100 ml of distillate was collected into 25 ml boric acid indicator solution.

3.4.5.3 Titration

The distillate was titrated with standard 0.02 N sulphuric acid and mixed using magnetic stirrer until the green colour solution turned pale lavender.

3.4.5.4 Calculation

mg NH_3 – N/ L = [(A-B) X 280] / ml sample X Dilution factor (Equation 3.3)

where,

A = volume of sulphuric acid titrated for the sample to turn to pale lavender color

B = volume sulphuric acid titrated for the blank to turn to pale lavender color

Figure 3.7 is a flow chart simplifying the experimental steps done. First step is sample collection and preparation and then the preparation of mixed culture; food waste, sludge and POME. Next step is experimental design and procedure using RSM method. After running the experiment, analysis of physical properties and also biogas were done.

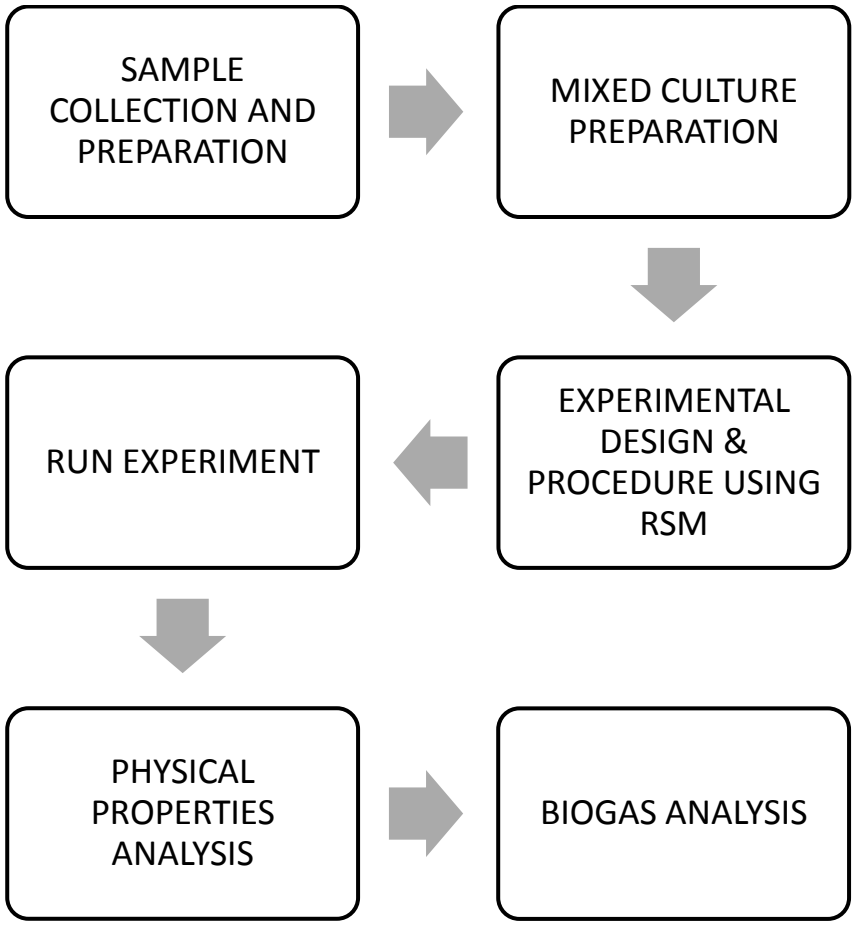

Figure 3.7: Flow chart of experimental steps

4.0 RESULTS AND DISCUSSIONS

4.1 Chemical Characterization

The chemical characteristics for food waste, sewage sludge and palm oil mill effluent (POME) used in this study is presented in Table 4.1. POME has the highest TSS, VSS, COD and TKN compared to food waste and sewage sludge. The pH for both food waste and POME was in the range of 4.0 to 4.5, while the sewage sludge obtained from anaerobic digester showed higher pH of 7.25 to 7.50. pH of food waste and POME are both acidic due to the by-product of nutrient degrading microorganism present in those source. Food waste has the lowest TKN because only a small amount of protein source was present. Food waste collected in this study consists mainly of rice while the remainder were vegetables, fish and meat.

Table 4.1: Chemical analysis of food waste, sewage sludge and POME

Analysis	Food waste	Sewage sludge	POME
TSS	762.7 mg/L	284 mg/L	833.2 mg/L
VSS	526.7 mg/L	244 mg/L	567.5 mg/L
COD	194625 mg/L	242000 mg/L	320040 mg/L
TKN	260.4 mg/L	492.8 mg/L	554.4 mg/L
pH	4.0 - 4.3	7.25 - 7.50	4.2 – 4.5

4.2 Cumulative Hydrogen Production

The biogas composition was determined using a gas chromatograph (Perkin Elmer, AutoSystem Gas Chromatography) equipped with thermal conductivity detectors (TCD) and digital data acquisition system. Hydrogen content was analyzed by GC-TCD fitted with a 1.5 m stainless steel column packed with a molecular sieve (80/100 mesh) the temperatures of injection port, oven and detector were 80°C, 200°C and 200°C respectively. The carrier gas used was Argon at a flow rate of 30 ml/ min. The gas sample was injected 1ml in replicates.

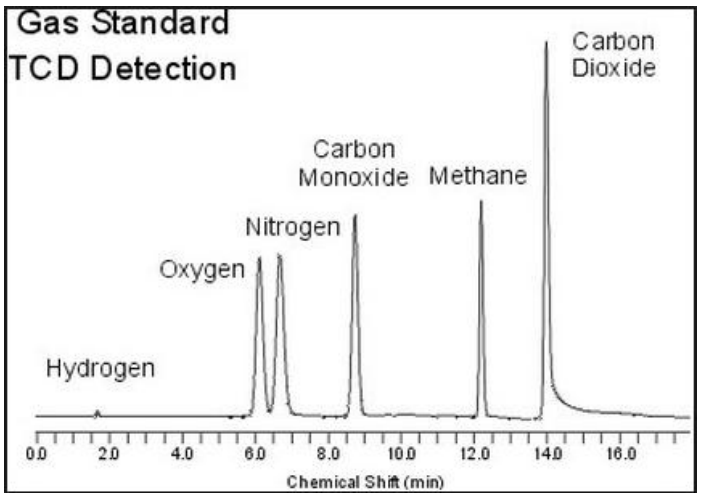

Figure 4.1: An example of chromatogram obtained using Perkin Elmer GC with TCD

Hydrogen gas production was calculated from headspace measurement of gas composition and the total volume of hydrogen produced using the mass balance equation as in Equation 3.1:

$$V_{Hi} = V_{Hi-1} + C_{Hi}(V_{Gi} - V_{Gi-1}) + V_{H0}(C_{Hi} - C_{Hi-1}) \qquad \text{(Equation 3.1)}$$

This mass balance equation was modified for the purpose of this study. Since cumulative hydrogen production is an increasing value, any sample reading (V_{Hi}) that showed decrease was neglected. This way, the cumulative hydrogen production remains a positive value.

4.3 Comparison between Anaerobic and Facultative Anaerobic Bacteria for Hydrogen Production

Anaerobic condition is established by sparging the serum bottles with oxygen-free nitrogen gas for 3 minutes at 20 ml/ minute. Facultative anaerobic condition for the experiment was obtained by sealing the serum bottles without any nitrogen gas sparging.

4.3.1 Effect of initial pH

The effect of initial pH was tested at 3 levels; pH 4.5, pH 5.5 and pH 6.5 while the temperature and inoculum size was kept constant at 35°C and 20%, respectively. At initial pH 4.5, aerobic system showed final cumulative hydrogen production of 7.21 ml while anaerobic system showed an average of final cumulative hydrogen production of 11.21 ml (Figure 4.2). At initial pH 5.5 the anaerobic system produces 9.11 ml of hydrogen and 4.38 ml of hydrogen was produced in the aerobic system (Figure 4.3). At initial pH 6.5, 9.05 ml of hydrogen was produced in the anaerobic system and 4.56 ml of hydrogen in the aerobic system (Figure 4.4).

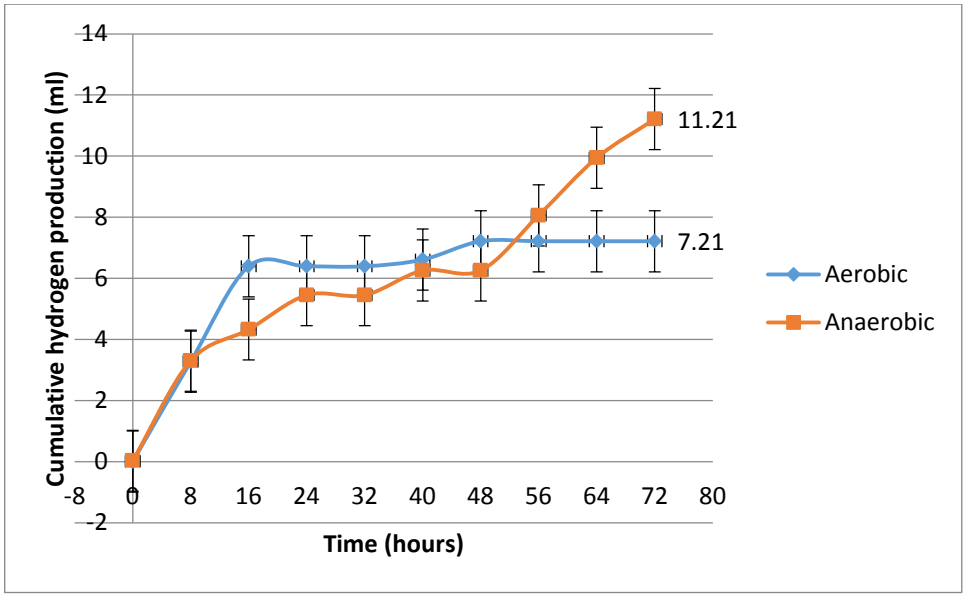

Figure 4.2: Cumulative hydrogen production for aerobic and anaerobic condition at initial pH 4.5

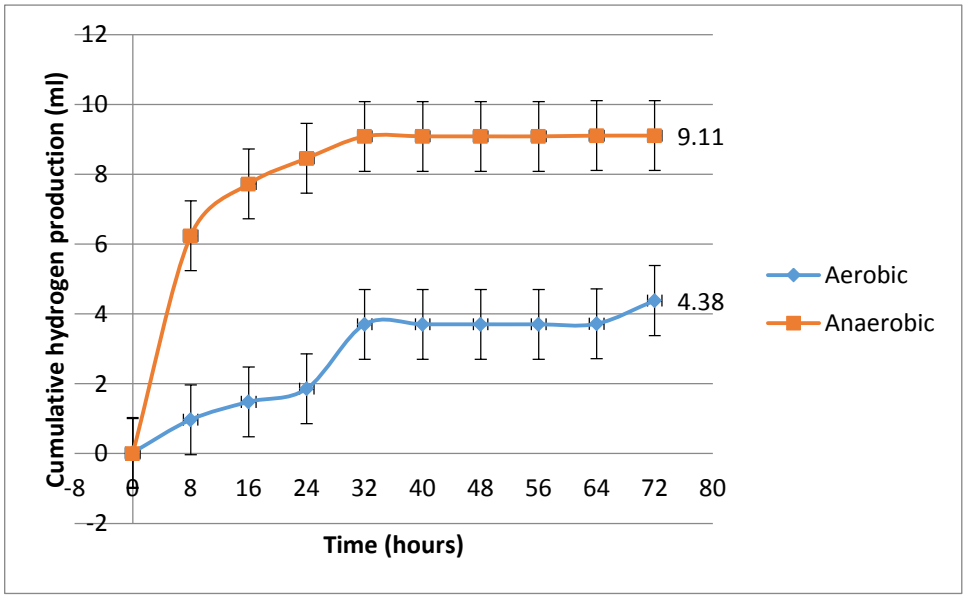

Figure 4.3: Cumulative hydrogen production for aerobic and anaerobic condition at initial pH 5.5

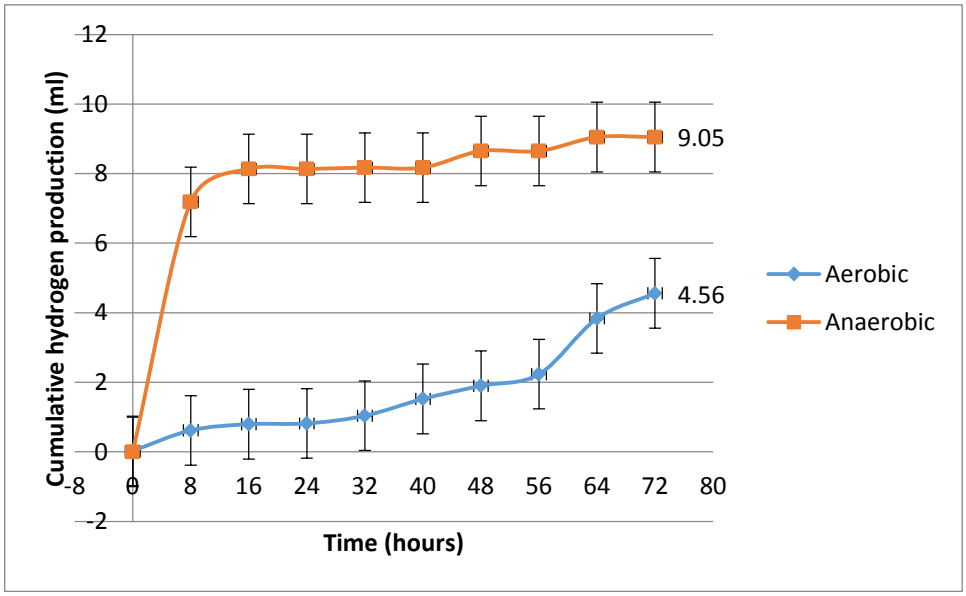

Figure 4.4: Cumulative hydrogen production for aerobic and anaerobic condition at initial pH 6.5

In general, all three pH conditions showed a gradual increase and eventually the production levelled off towards the end of fermentation for both anaerobic and aerobic

systems. However, the hydrogen gas production slope of anaerobic system was much steeper i.e. the rate of production was higher than in aerobic system.

In this study, the anaerobic system showed higher cumulative hydrogen gas production compared to aerobic system for all three initial pH tested. However, the production level of these two systems was not significantly different at pH 4.5 and 5.5. At initial pH 6.5, however, the production level in anaerobic system was almost 50% higher than in aerobic system.

Within appropriate range, increase in pH is directly related to increased hydrogen production. However, if pH is too high, microbial hydrogen production ability will be decreased (Wang and Wan, 2009). A similar study done by Fang *et al* (2006) investigates the effect of initial pH in the range of 4.0 to 7.0 using a batch reactor. The optimal initial pH obtained was pH 4.5 with maximum hydrogen yield of 346 ml/ g starch. In another study by Khanal *et* al (2004), in the range of 4.5 to 6.5 the optimal initial pH was also found to be pH 4.5.These results agreed with the finding of this study that showed initial pH 4.5 is optimum based on the highest cumulative hydrogen obtained.

4.3.2 Effect of temperature

The effect of temperature on cumulative hydrogen production was tested at 3 levels *viz.* 35°C, 45°C and 55°C while the initial pH and inoculum size was kept constant for all parameters at 4.5 and 20%, respectively. At 35°C, anaerobic system has the cumulative hydrogen gas production of 8.52 ml while anaerobic system has an average of cumulative hydrogen gas production of 7.91 ml (Figure 4.5). At 45°C the anaerobic system produced 6.97 ml of hydrogen gas while 6.23 ml of hydrogen gas was produced in the aerobic system (Figure 4.6). At 55°C, 7.30 ml of hydrogen gas was

produced in the anaerobic system while 5.42 ml of hydrogen was obtained in the aerobic system (Figure 4.7).

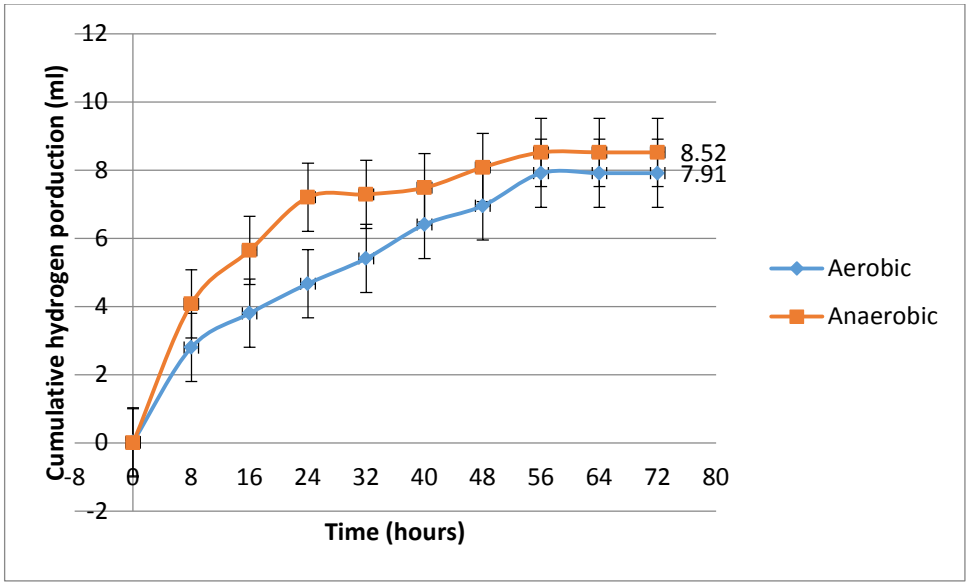

Figure 4.5: Cumulative hydrogen production for aerobic and anaerobic condition at 35°C

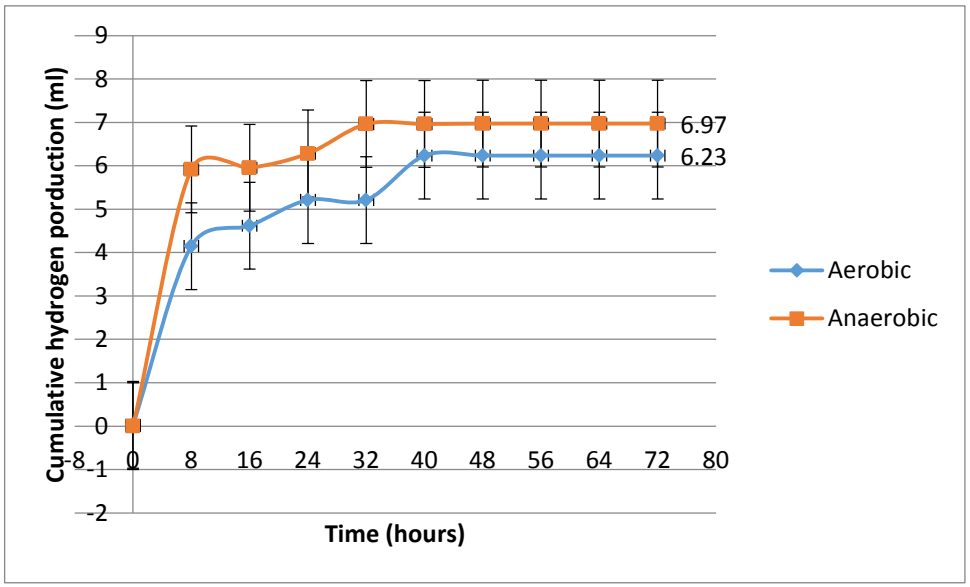

Figure 4.6: Cumulative hydrogen production for aerobic and anaerobic condition at 45°C

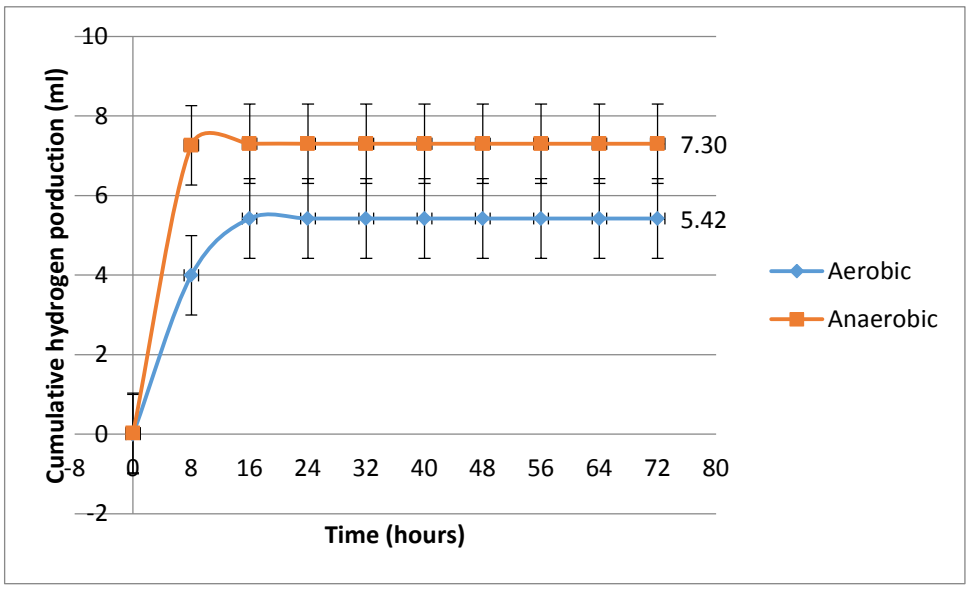

Figure 4.7: Cumulative hydrogen production for aerobic and anaerobic condition at 55°C

At all three temperature levels there were increasing cumulative hydrogen gas production and eventually the amount remained constant towards the end of fermentation for both systems. However, anaerobic system showed slightly higher rate of production than facultative anaerobic system.

At 45°C and 55°C, hydrogen production rate was faster and reached stationary phase sooner. At 35°C, the hydrogen gas production only started to level off at 56th hour, while at 45°C the stationary phase started at 40th hour. The stationary phase for 55°C started even earlier at 16th hour for anaerobic system and 24th hour for aerobic system.

A higher temperature speeds up the reaction rate, which is beneficial for a larger scale hydrogen production since reduced production time contributes to reduced cost. However, a temperature that is above optimal may suppress hydrogen producing microbes thus reducing hydrogen yield (Wang and Wan, 2008). Although the anaerobic system showed higher cumulative hydrogen production compared to aerobic system for

all the temperature conditions tested, the final hydrogen gas production of these two systems were not much different.

Even though the optimal temperature reported for microbial hydrogen production studies may differ, most fell into the range of mesophilic and thermophilic range, depending on the type of substrate and inoculum used (Wang and Wan, 2009). Li *et al* (2007) reported that 73 out of 101 case studies were carried out at mesophilic temperatures. Studies done by Shin *et al* (2004) and Kim *et al* (2004) both showed optimal temperature in mesophilic range for hydrogen gas production with specific hydrogen production rate (SHPR) of 12 ml/ g VSS h and 0.7 ml/ g VSS h, respectively.

4.3.3 Effect of inoculum size

The effect of inoculum size was tested at three levels, 2%, 11% and 20%. When the inoculum size was set at 2% volume/ volume (ml) the anaerobic system showed final cumulative hydrogen gas production 5.02 ml while the aerobic system 4.32 ml as shown in Figure 4.8. Similar observation was made for 11% inoculum size (Figure 4.9). At inoculum 20%, both systems reached stationary phase at approximately 40^{th} hour. However, the difference in cumulative hydrogen gas production for both the anaerobic and aerobic system is small *i.e*. 6.97 ml and 5.42 ml respectively (Figure 4.10).

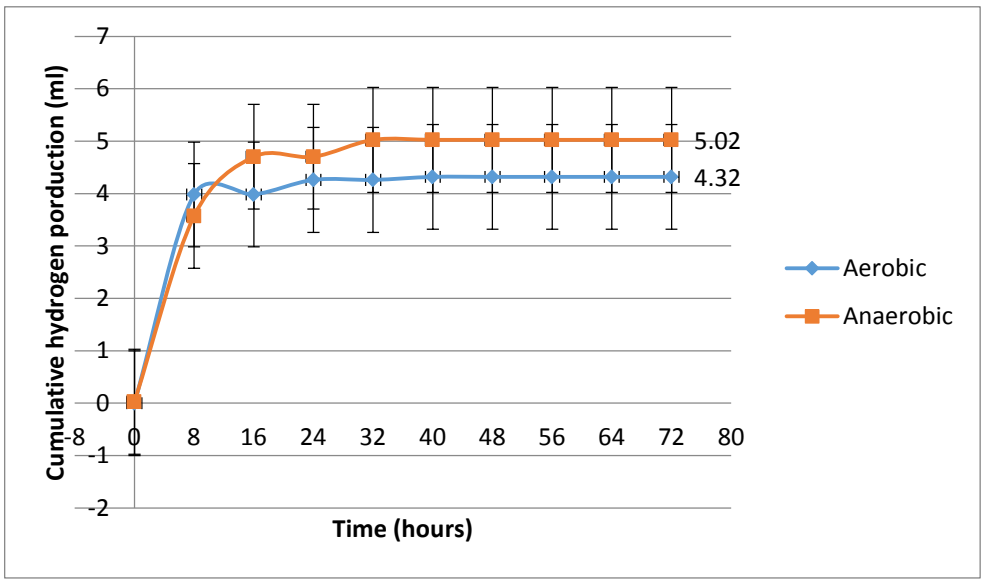

Figure 4.8: Cumulative hydrogen production for aerobic and anaerobic condition at inoculum size 2%

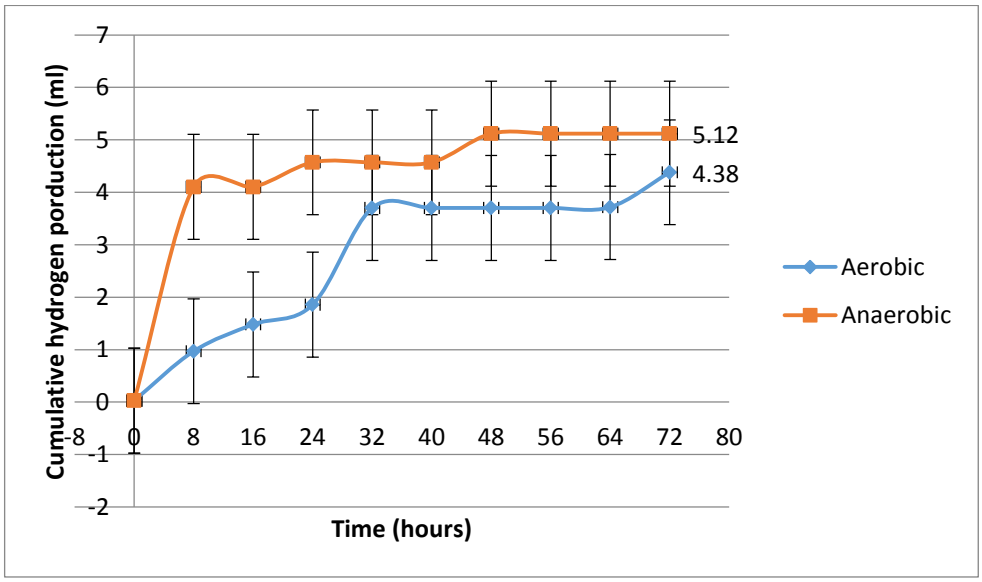

Figure 4.9: Cumulative hydrogen production for aerobic and anaerobic condition at inoculum size 11%

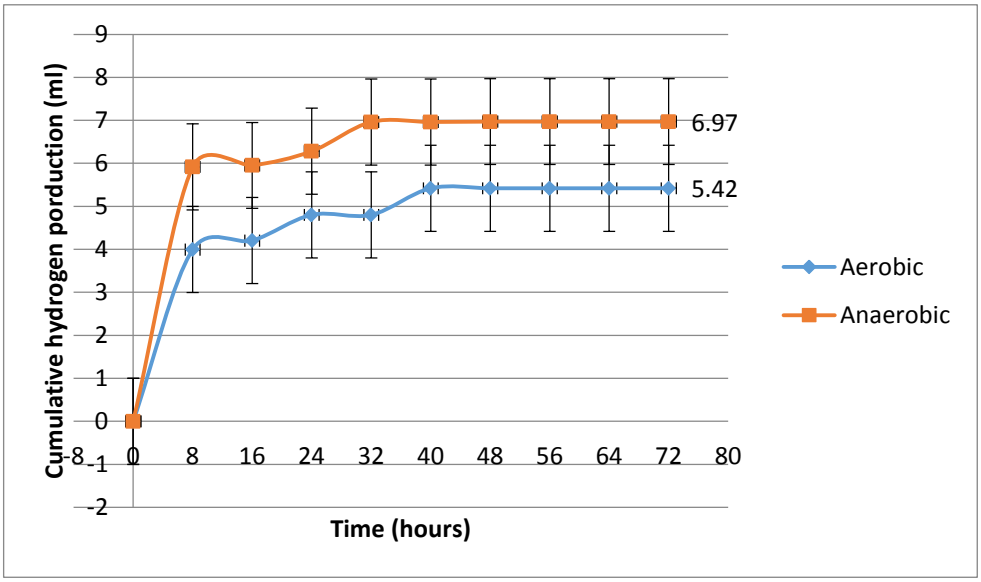

Figure 4.10: Cumulative hydrogen production for aerobic and anaerobic condition at inoculum size 20%

The inoculum size showed significant effect on the cumulative hydrogen gas production of the systems tested. Higher inoculum size produces higher cumulative hydrogen production. A logical explanation behind this is that the production of hydrogen gas was directly related to the number of hydrogen producing organisms that exist in the system.

It is obvious that for all three conditions tested, higher hydrogen gas production was shown by anaerobic system in relative to facultative anaerobic. This indicated that majority of the microbial population in the mixed culture were obligate anaerobes. The microbial DNA profiling data in study by Fang *et al* (2006) indicated that the mixed culture microbes from sewage sludge were mainly made up of *Clostridium* sp; a Gram negative rod shaped obligate anaerobes and spore forming bacteria.

Hydrogen production in the liquid phase can be strongly affected by the hydrogen gas partial pressure. The key enzyme involved is hydrogenase; it reversibly oxidizes and reduces ferrodoxin. When the hydrogen concentration is high in liquid

phase, reduction of oxidized ferrodoxin will take place instead of oxidation of reduced ferredoxin. In other word, hydrogen in liquid phase will oxidize to proton, therefore reducing the yield of hydrogen (Chong *et al*, 2009).

Gas sparging with inert gasses such as nitrogen is normally done to create an anaerobic condition. It is also as an alternative to reduce hydrogen partial pressure. The main disadvantage of gas sparging is that it dilutes the hydrogen content and creates further reduction in efficiency of separation. In larger scale, this technique will require high energy consumption for sparging process and downstream processing of hydrogen purification which in turn will increase the production cost. Sparging industrial size reactors is feasible when the sparging gas is produced onsite. If the sparging gas is the same composition as the headspace gas, this technique is simply another form of mixing.

In a study by Kim et al (2006), there does not seem to be any relationship between amount of sparging and increase in hydrogen yield when multiple sparging rates were tested to compare hydrogen yield with increasing rate of sparging. However, sparging did improve hydrogen yield than unsparged condition. Thus, it was concluded that hydrogen production can be significantly improved by sparging gas into the system.

Clark *et al* (2012) in their study has shown that sparging with nitrogen and carbon dioxide increased the specific hydrogen production rate and final yield. The highest yield obtained was 1.68 mol hydrogen/ mol of hexose when sparged with carbon dioxide at 300 ml/ minute. Normally higher hydrogen yields will be accompanied by high acetate production. However, 16S rRNA analysis of the microbial community showed that carbon dioxide sparging inhibits the homoacetogens causing lower acetate production and hydrogen consumption.

Hussy *et al* (2003) examined the effect of nitrogen gas sparging on hydrogen production in a continuously stirred tank reactor (CSTR) digesting wheat flour industry byproducts. Sparged system showed an increase in hydrogen production from 1.3 to 1.9 mol hydrogen/ mole hexose. Mizuno *et al* (2000) used a mixed culture grown in glucose in a CSTR that was also sparged with nitrogen gas. They reported increased yields from 0.85 to 1.43 mole hydrogen/ mole glucose with no major shift in fermentation products occurred.

4.4 Response Surface Methodology (RSM)

In this work, RSM was used to determine the optimal points of temperature, inoculum size and pH for biohydrogen production from 1) food waste with a mixed culture from pre-treated sludge (Production 1) and 2) mixture of food waste and palm oil mill effluent (POME) with a mixed culture from pre-treated sludge (Production 2).

For each parameter studied, three points were chosen; a minimum, middle and maximum point. The parameters and their respective points were: Temperatures of 35°C, 45°C and 55°C; Inoculum sizes of 2%, 10% and 20% volume/volume (ml); pH of 4.5, 5.5 and 6.5. These parameters' levels and symbols are shown in Table 4.2.

Factors that influence the hydrogen gas production were evaluated using factorial plots, main effects plots, interaction effects, normal probability plots and contour plots. The ANOVA and *p*-value significant levels were used to check the significance of the variables on hydrogen gas production.

Table 4.2: Symbols for variables and their levels

Symbols	Variables	Levels		
A	Initial pH	4.5	5.5	6.5
B	Temperature (°C)	35	45	55

C	Inoculum size (% volume/ volume) (ml)	2	11	20

4.4.1 Analysis of Result for Cumulative Hydrogen Gas Production for Food Waste as Sole Substrate (Production 1)

The multiple regression analysis done using Minitab 16.1 software showed the importance of main and interaction effects of the three variables (Table 4.3).

Table 4.3: Estimated effects and coefficients for hydrogen production from food waste, Production 1 (coded units)

Term	Effect	Coef	SE Coef	*T*	*P*
Constant		6.5965	0.1902	34.68	0.000
Ph	-0.1926	-0.0963	0.2018	-0.48	0.638
Temperature	-1.9980	-0.9990	0.2018	-4.95	0.000
Inoculum	0.9458	0.4729	0.2018	2.34	0.030
pH*Temperature	1.3856	0.6928	0.2018	3.43	0.003
pH*Inoculum	0.1291	0.0645	0.2018	0.32	0.752
Temperature*Inoculum	0.2580	0.1290	0.2018	0.64	0.530
pH*Temperature*Inoculum	-0.0274	-0.0137	0.2070	-0.07	0.948

S = 0.98843 *PRESS* = 35.3015
R^2 = 68.03%

Table 4.4: Analysis of variance for hydrogen produced from food waste, Production 1 (Coded units)

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Main Effects	3	29.5414	29.5414	9.8471	10.08	0.000
pH (A)	1	0.2225	0.2225	0.22	0.23	0.638
Temperature (B)	1	23.9518	23.9518	23.9518	24.52	0.000
Inoculum (C)	1	5.3671	5.3671	5.3671	5.49	0.030
2-Way Interactions	3	12.0181	12.0181	4.0060	4.10	0.020
pH*Temperature (AB)	1	11.5189	11.5189	11.5189	11.79	0.003
pH*Inoculum (AC)	1	0.1000	0.1000	0.1000	0.10	0.752
Temperature*Inoculum (BC)	1	0.3992	0.3992	0.3992	0.41	0.530
3-Way Interactions	1	0.0045	0.0045	0.0045	0.00	0.948
pH*Temperature*Inoculum (ABC)	1	0.0045	0.0045	0.0045	0.00	0.948
Residual Error	20	19.5404	19.5404	0.9770		
Curvature	1	3.7451	3.7451	3.7451	4.50	0.047
Lack of Fit	1	0.0045	0.0045	0.0045	0.01	0.944
Pure Error	18	15.7908	15.7908	0.8773		

Total	26	61.0999

Both Table 4.3 and 4.4 are obtained directly from Minitab 16.1 software. The multiple regression analysis done using the software showed the importance of main and interaction effects of the three variables (Table 4.4). In order to simplify the calculation, coded variables are used for describing independent variables in the (-1, 1) interval; -1 represents the low settings while 1 are the high settings. Minitab uses coded units to allow comparison of coefficients' sizes to determine which factor has the largest impact on the response (Bradley, 2007).

The significance of the regression coefficients was determined by applying a *t*-test. The temperature and inoculum size (volume/volume) (ml), showed significant effect on the cumulative hydrogen production, where $P < 0.05$. The initial pH chosen for this study, ranging from 4.5 to 6.5 did not show any significance; probably due to the small range tested. However, the two-way interaction effect between initial pH and temperature was significant ($P = 0.003$).

The coefficient of multiple determinations, R^2 shows how well the estimated model fits the data. The closer R^2 is to 1, the better the model fits the experimental data. The value of R^2 for this part is 0.6802. This means that the model could explain 68.02% of the total variation in the system.

The predicted value of response was obtained from full quadratic model fitting technique which includes the main effects and interaction effects. The regression equation generated by Minitab software in coded unit is given in Equation (4.1):

$$\boldsymbol{Y = 6.5965 - 0.0963A - 0.9990B + 0.4729C + 0.6928AB + 0.0645AC + 0.1290BC - 0.0137ABC} \quad \text{(Equation 4.1)}$$

Where *Y* (yield) is the cumulative hydrogen in ml; *A* is initial pH, *B* is temperature (°C) and *C* is inoculum size (% v/v). The ANOVA analysis for Equation 4.1 is shown in Table 4.4.

The function in Equation (4.2) describes how the experimental variables and their interactions influence the hydrogen production. The initial pH of the system (*A*) had the greatest effect on hydrogen production, followed by temperature (*B*), inoculum size (*C*) and initial pH-temperature interaction (*AB*). The positive values of these effects showed that the increase in these parameters should result in the increase of response, in this case hydrogen production. The negative values of the effects on the other hand will result in decreased response. According to Equation (4.2), the initial pH and temperature showed negative effect on cumulative hydrogen production while inoculum size and all the interaction effects showed positive effect on hydrogen production.

The main effects of each parameter on cumulative hydrogen production are showed in Figure 4.11. This main effect plot was generated to represent the results of the regression analysis. It showed deviations of the average between the high and low levels of each factor. When the effect of a factor to the response is positive, the response increases from low level to high level of that factor. Conversely, when the factor gives negative effect, the response will decrease from a high level to low level.

From Figure 4.11, the steeper the response line, the larger is the change in response value (y) when changing from coded level -1 to level +1. The statistical significance of a factor is directly related to the length of the line (Palanikumar and Dawim, 2009). The initial pH and temperature both showed negative effects on hydrogen production. However, temperature factor exerted larger effect which was evident by longer line and steeper slope compared to that of initial pH. Inoculum size on the other hand, showed a positive effect on hydrogen production.

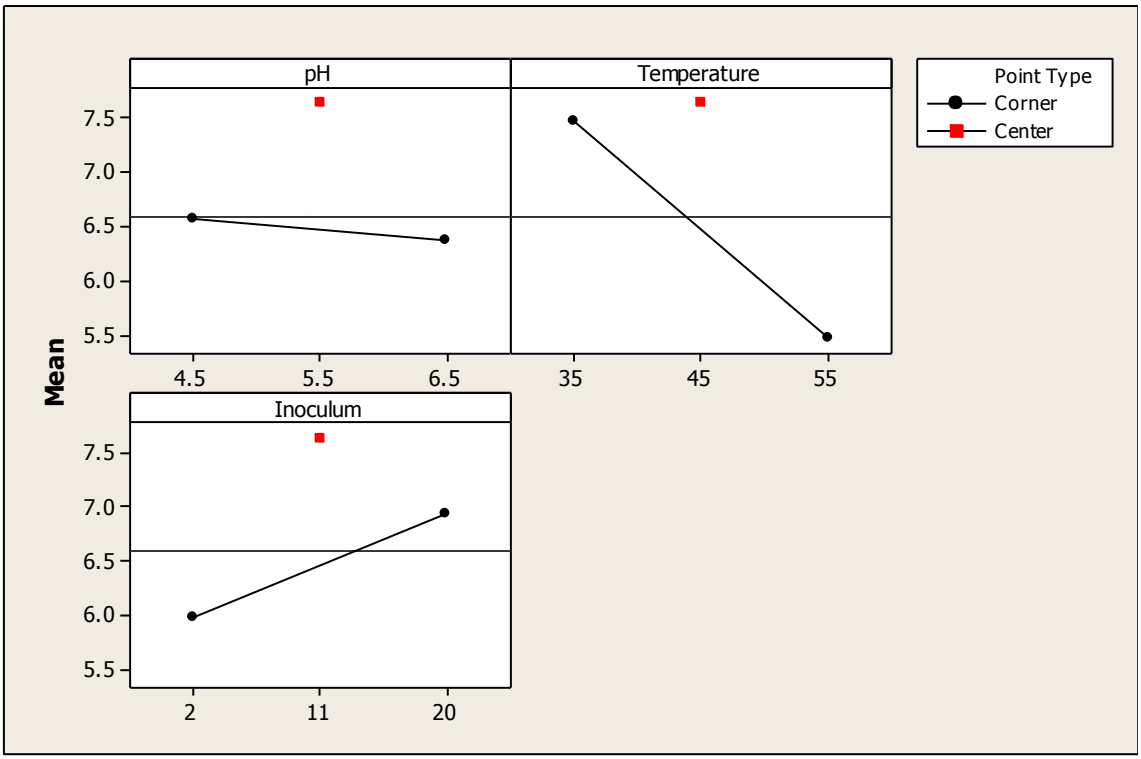

Figure 4.11: Main effects plot for hydrogen production for Production 1

Figure 4.12 represents the effectiveness of interaction between factors. The change in response from low to high levels of those factors depends on the level of a second factor, that is whether or not the lines run parallel to each other (Mathialagan and Viraraghavan, 2005). It should be pointed out that this interaction effects plot was also generated from ANOVA analysis and from the result in Table 4.5, only initial pH-temperature interaction (*AB)* has significant effect on cumulative hydrogen gas production. This is obvious from the crossing of the response line for both initial pH and temperature. The interactions of initial pH-inoculum size and temperature-inoculum were insignificant as shown by the lines running parallel to each other.

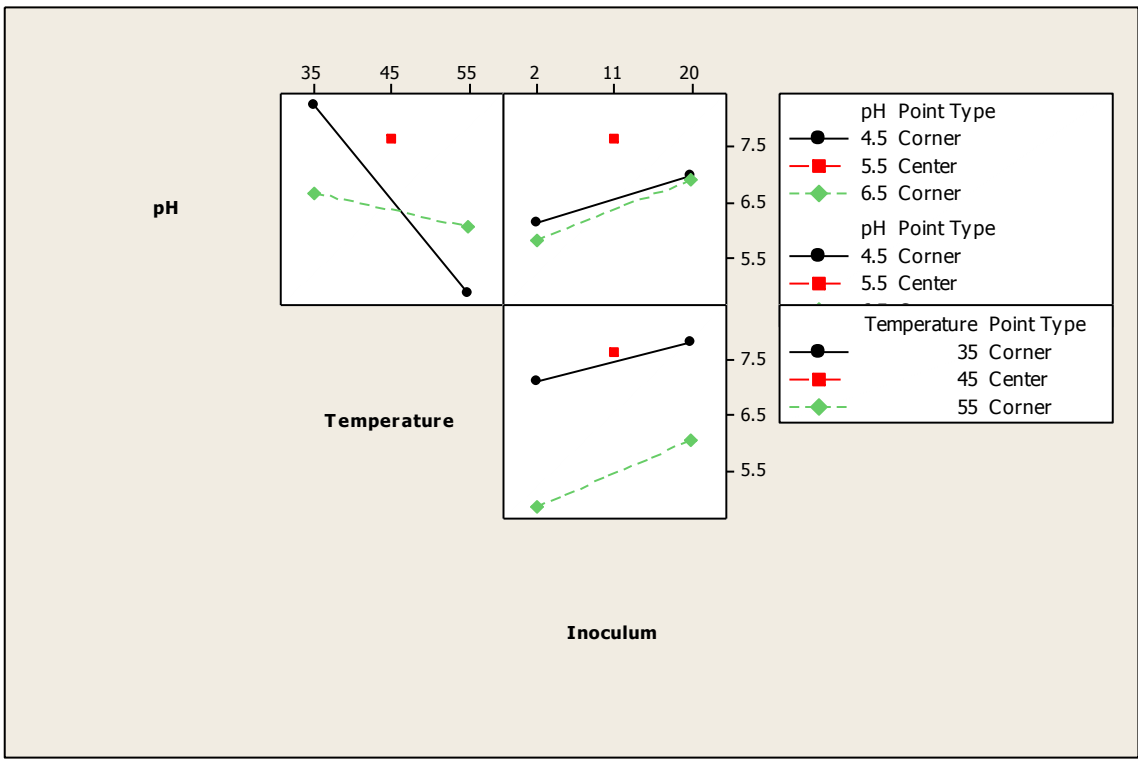

Figure 4.12: Interaction plot for hydrogen produced in Production 1 (Food waste only)

The standardized residual plots for hydrogen gas production in Production 1 is shown in Figure 4.13. The normal probability plot showed that normality assumption was satisfied for the experimental data. Most of the data points on the plot fell closely to the normal line (Velickovic *et al*, 2013).

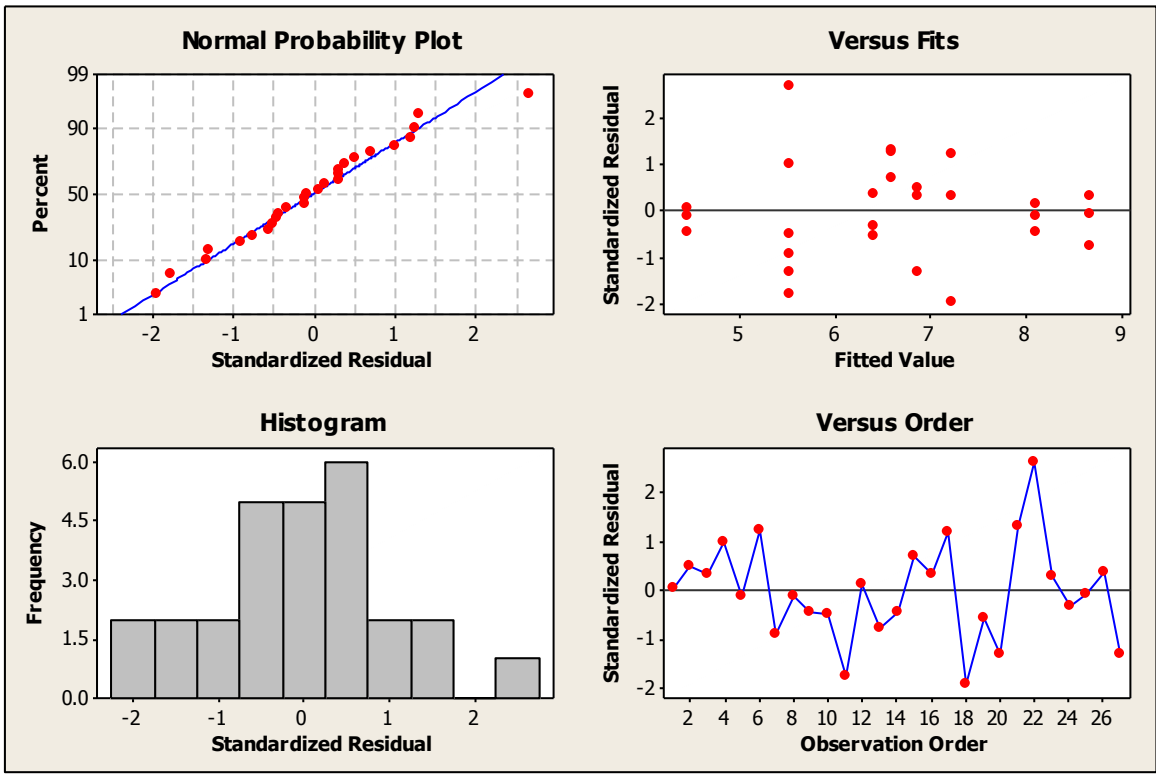

Figure 4.13: Residual plots for hydrogen production in Production 1

Normal probability plots determine whether or not the results obtained are reliable or just by chance. The points that are close to a line fitted to the middle group of points represents factors that do not have significant effect on the response (Bingol *et al*, 2010). In Figure 4.14, only the main factors temperature (*B*) and inoculum size (*C*) were significant, so did interaction of initial pH and temperature (*AB*) (all marked with red squares). The inoculum size (*C*) and initial pH-temperature (*AB*) lies on the right side of the line means that they have positive effect on the response. The main factor temperature (*B*) lies on the left side showing its negative effect upon the response that is cumulative hydrogen ga production. The effects in decreasing manner is $B > AB > C$; decided by looking at the distance of the points from the line.

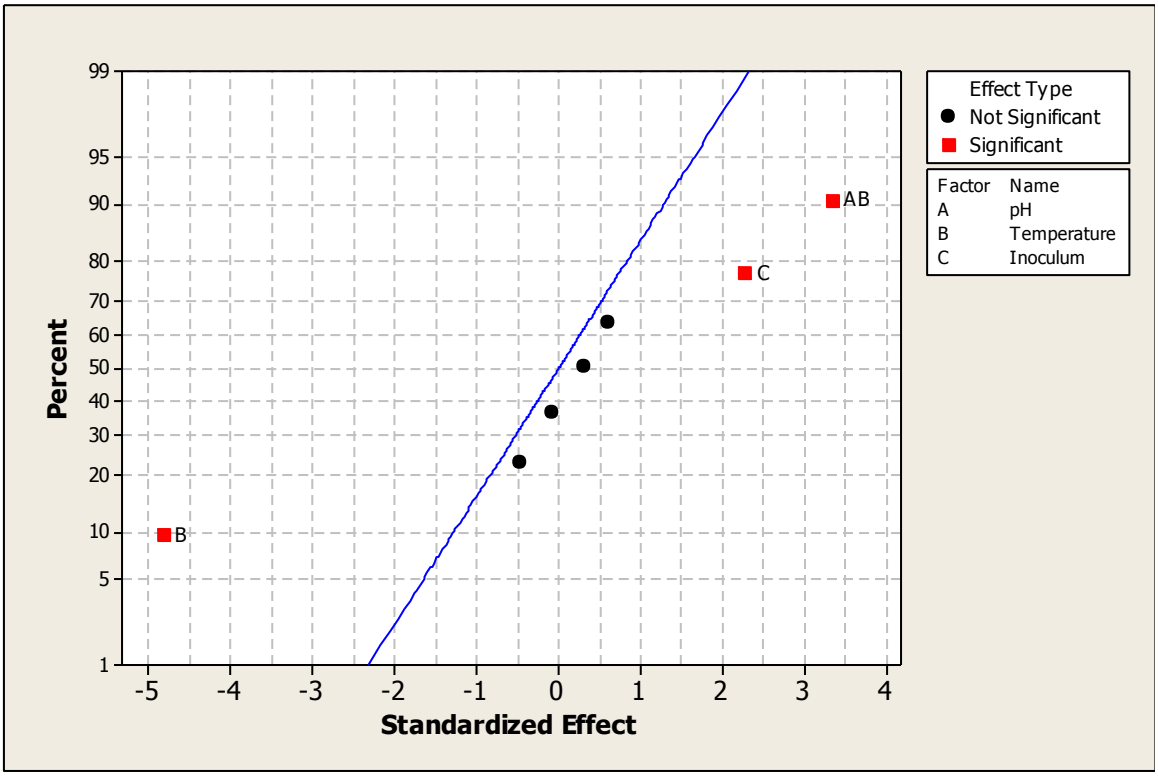

Figure 4.14: Normal plot of the standardized effects for Production 1

Contour plots are generated to better understand both the main and interaction effects of the factors in an experiment. Contour plots have curved lines since this model showed the interactions between the factors (*AB*, *AC* and *BC*), when one of the parameters for each graph is at a constant value.

In Figure 4.15, the curvature showed the significance of the temperature and initial pH interaction. This is supported by the result of ANOVA in Table 4.5, where *AB* interaction has *p* value of 0.003. The arrow shows the trajectory of optimization, and in this case it is towards lower initial pH and lower temperature.

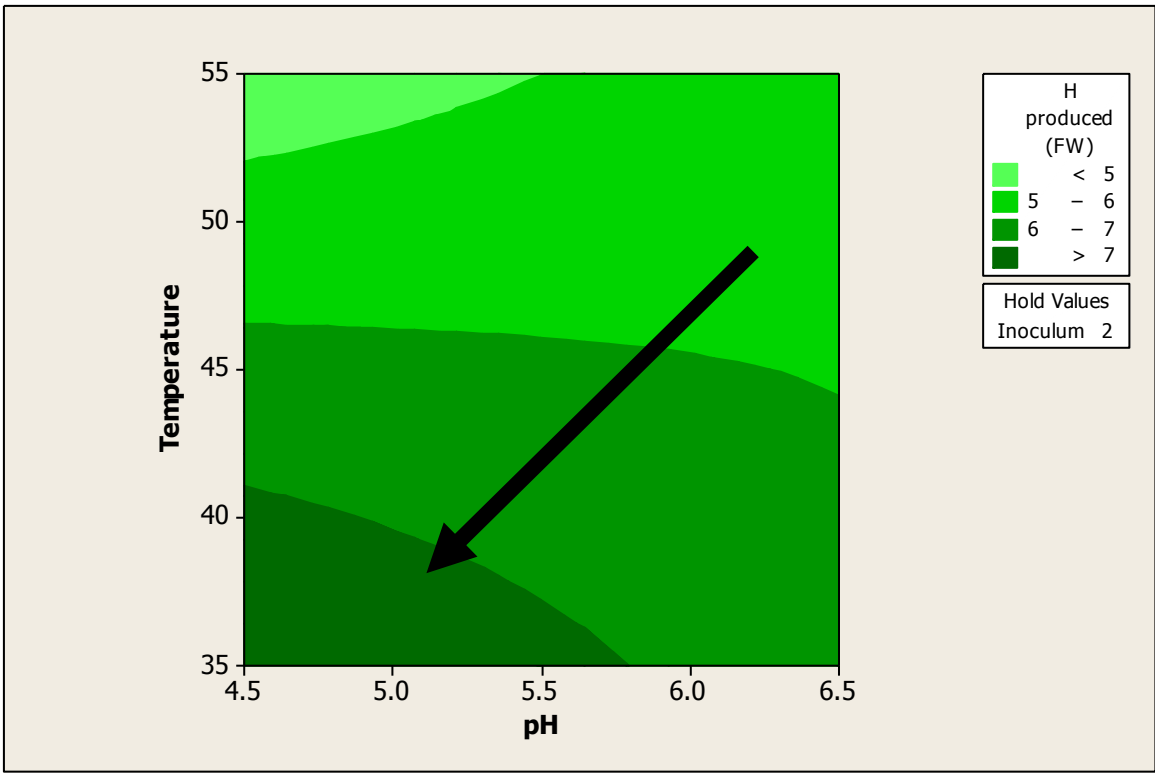

Figure 4.15: Contour plot of hydrogen production versus temperature and initial pH from food waste (Production 1)

Figure 4.16 showed the result of *AB* interaction in three dimensions. The highest point of maximum response is at low levels of initial pH and temperature.

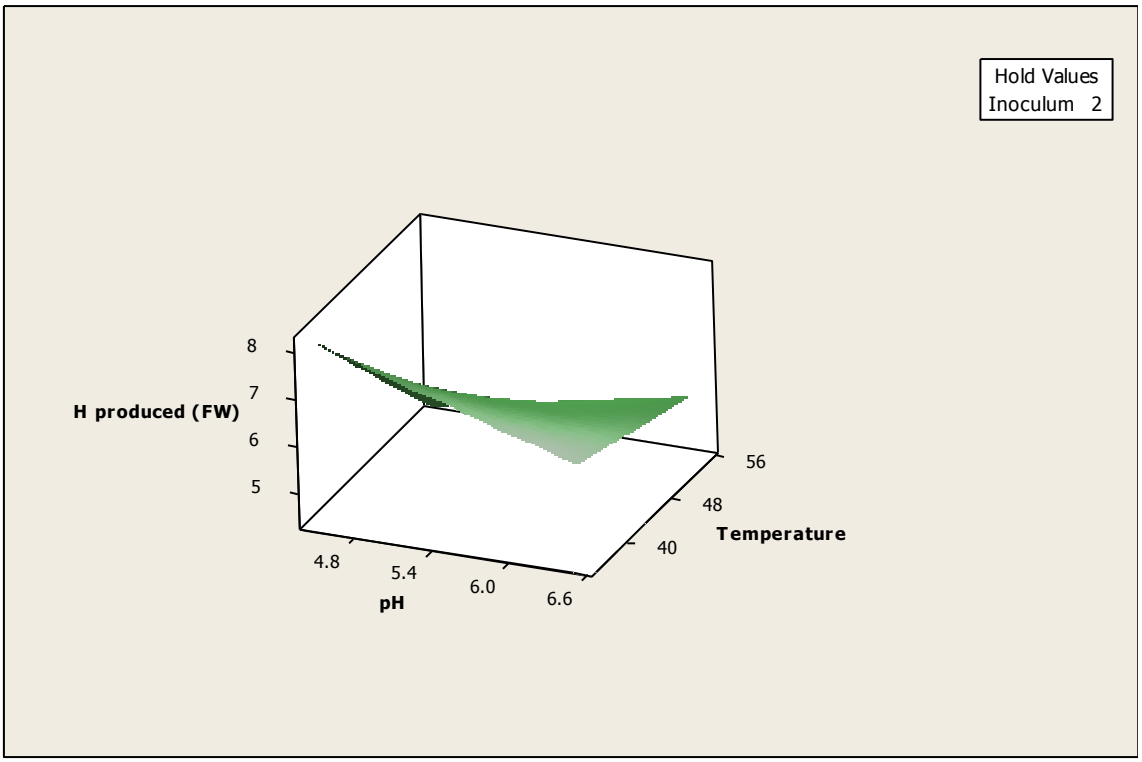

Figure 4.16: 3D surface plot of hydrogen production versus temperature (B) and initial pH (A) from food waste (Production 1)

It can be concluded from Figure 4.17 and 4.18 that there were no significant interaction between the other two factors *i.e.* time *AC* and *BC*, judging from the almost linear contour. This was also verified from Table 4.5, where the interaction effects showed $P > 0.05$.

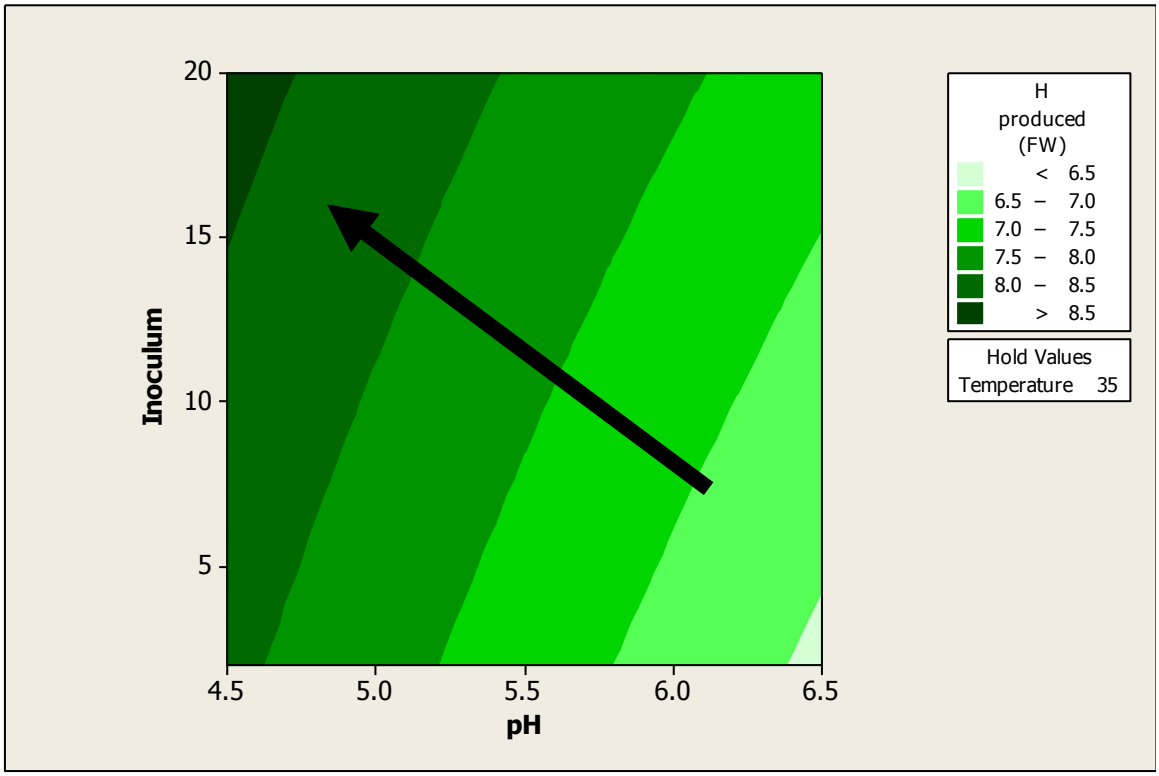

Figure 4.17: Contour plot of hydrogen production versus inoculums size and initial pH from food waste (Production 1).

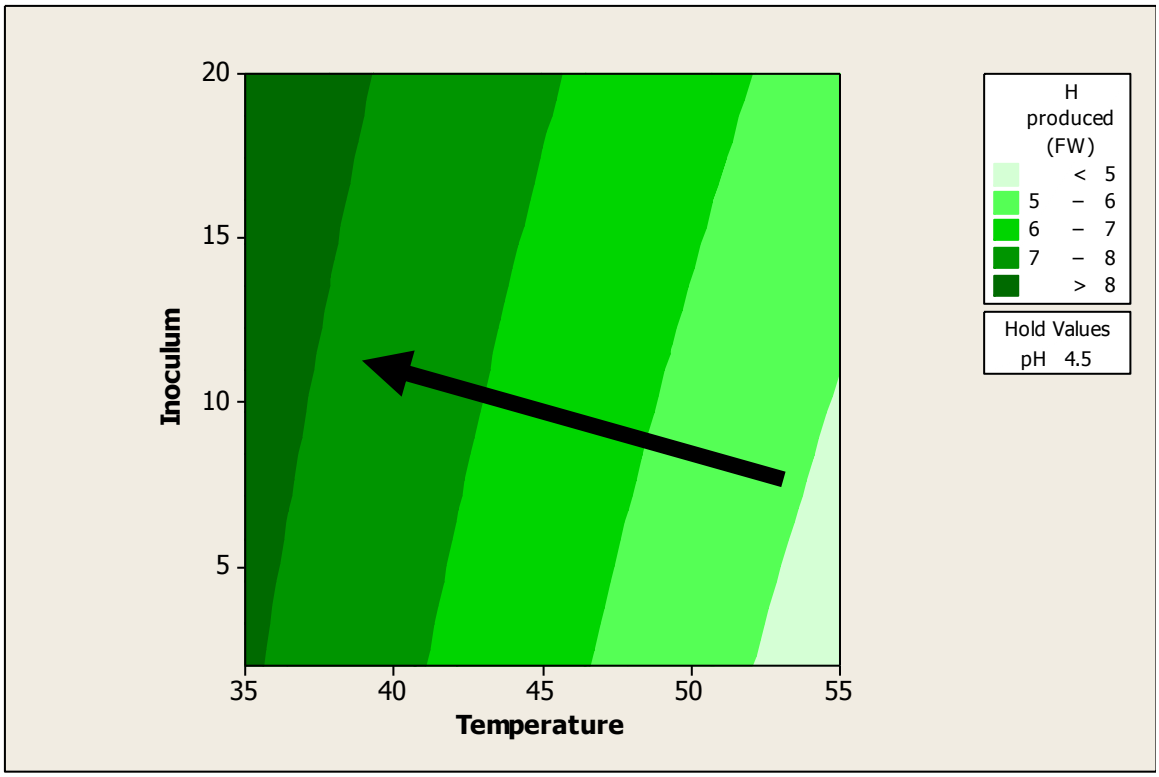

Figure 4.18: Contour plot of hydrogen production versus inoculum size and temperature from food waste (Production 1).

Figure 4.19 is a cube plot that enables us to see the predicted responses based on a three way interactions of the factors *A*, *B* and *C*. For Production 1, highest response would be at lowest initial pH (*A*), lowest temperature (*B*) and highest inoculum size (*C*).

From the response optimizer function in Minitab, the maximum predicted hydrogen production (MPHP) of 9.74 ml can be obtained with these parameters set; initial pH of 4.5, inoculum size of 20% and temperature of 35°C. This parameters set obtained was also in agreement with the finding presented in cube plot (Figure 4.18). The composite desirability obtained from Minitab software was 0.9338 which indicates that it is possible to obtain predicted results in 93 times out of 100 runs.

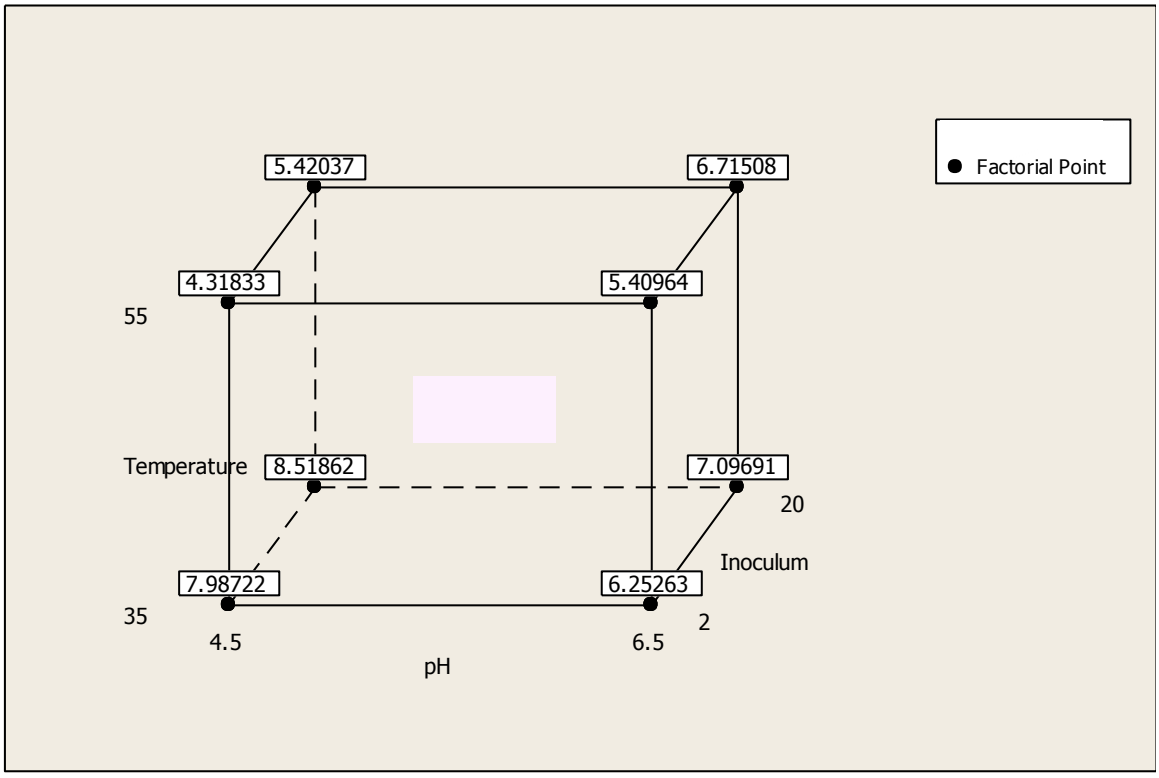

Figure 4.19: Cube plot for hydrogen production from food waste (Production 1)

4.4.2 Analysis of Result for Cumulative Hydrogen Production for Food Waste mixed with POME as substrate (Production 2)

Table 4.5: Estimated effects and coefficients for hydrogen production of substrate food waste mixed with POME, Production 2 (Coded units)

Term	Effect	Coef	SE Coef	*T*	*P*
Constant		7.951	0.1891	42.04	0.000
pH	-2.505	-1.253	0.2006	-6.24	0.000
Temperature	-1.369	-0.684	0.2006	-3.41	0.003
Inoculum	0.925	0.462	0.2006	2.30	0.033
pH*Temperature	-0.278	-0.139	0.2006	-0.69	0.497
pH*Inoculum	0.837	0.419	0.2006	2.09	0.051
Temperature*Inoculum	-0.787	-0.394	0.2006	-1.96	0.064
pH*Temperature*Inoculum	-2.832	-1.416	0.2006	-7.06	0.000

S = 0.9826 $PRESS$ = 27.0035

R^2 = 85.76%

Table 4.6: Analysis of variance for hydrogen produced from food waste mixed with POME, Production 2 (Coded units)

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Main Effects	3	54.022	54.0220	18.0073	18.65	0.000
pH	1	37.651	37.6514	37.6514	38.99	0.000
Temperature	1	11.240	11.2401	11.2401	11.64	0.003
Inoculum	1	5.131	5.1305	5.1305	5.31	0.033
2-Way Interactions	3	8.391	8.3905	2.7968	2.90	0.062
pH*Temperature	1	0.463	0.4628	0.4628	0.48	0.497
pH*Inoculum	1	4.207	4.2074	4.2074	4.36	0.051
Temperature*Inoculum	1	3.720	3.7204	3.7204	3.85	0.064
3-Way Interactions	1	48.108	48.1077	48.1077	49.82	0.000
pH*Temperature*Inoculum	1	48.108	48.1077	48.1077	49.82	0.000
Residual Error	19	18.348	18.3481	0.9657		
Curvature	1	12.384	12.3842	12.3842	37.38	0.000
Pure Error	18	5.964	5.9639	0.3313		
Total	26	128.868				

The multiple regression analysis using Minitab 16.1 software showed the importance of main effects and interaction effects of 3 variables based on the responses of maximum predicted cumulative hydrogen gas production from mixed substrate (Production 2). Table 4.5 showed the important effects of the variables on the cumulative hydrogen gas production.

The coefficient of multiple determinations, R^2 is 0.8576. This means that the model could explain 85.76% of the total variation in the system. PRESS and R^2 are essentially redundant. However the predicted R^2 can be compared directly to the regular R^2 for the model, to judge whether the latter accurately reflects the predictive value of the model or is inflated by over-fitting (Bradley,2007).

Based on Table 4.6, all three factors; initial pH, temperature and inoculum size volume/volume (mL), showed significant effect to the cumulative hydrogen gas production, where $P < 0.05$. However, the interaction effects between the factors did not show any significance. The three way interaction (*ABC*) somehow did show significance at $P = 0.000$.

The predicted value of response was obtained from full quadratic model fitting technique which includes the main effects and interaction effects. The regression equation generated by Minitab software is given in Equation 4.2:

$$Y = 7.951 - 1.253A - 0.684B + 0.462C - 0.139AB + 0.419AC - 0.394BC - 1.416ABC \quad \text{(Equation 4.2)}$$

Where *Y* (yield) is the cumulative hydrogen in ml; *A* is initial pH, *B* is temperature (°C) and *C* is inoculum size (% v/v). The ANOVA analysis for Equation 4.2 is shown in Table 4.6.

The function in Equation (4.3) explained the effects of the variables and their interactions on the response. From the equation, initial pH (*A*) showed the greatest effect on hydrogen production, followed by inoculum size (*C*) temperature (*B*) and then the initial pH – inoculum size interaction. The main effects *A*, *B* and interaction effects *AB* and *BC* have negative values which hence will decrease the cumulative hydrogen gas production. The main effect *C* and interaction effect *AC* have positive values indicating that these interactions positively affect the response.

Figure 4.20 showed the effects of each variable on cumulative hydrogen gas production. Only the inoculum size (*C*) has positive effect on the response, while the initial pH (*A*) and temperature (*B*) has negative effect. The initial pH (*A*) has the biggest effect on the response looking at its long vertical line and steeper slope (Palanikumar and Dawim, 2009).

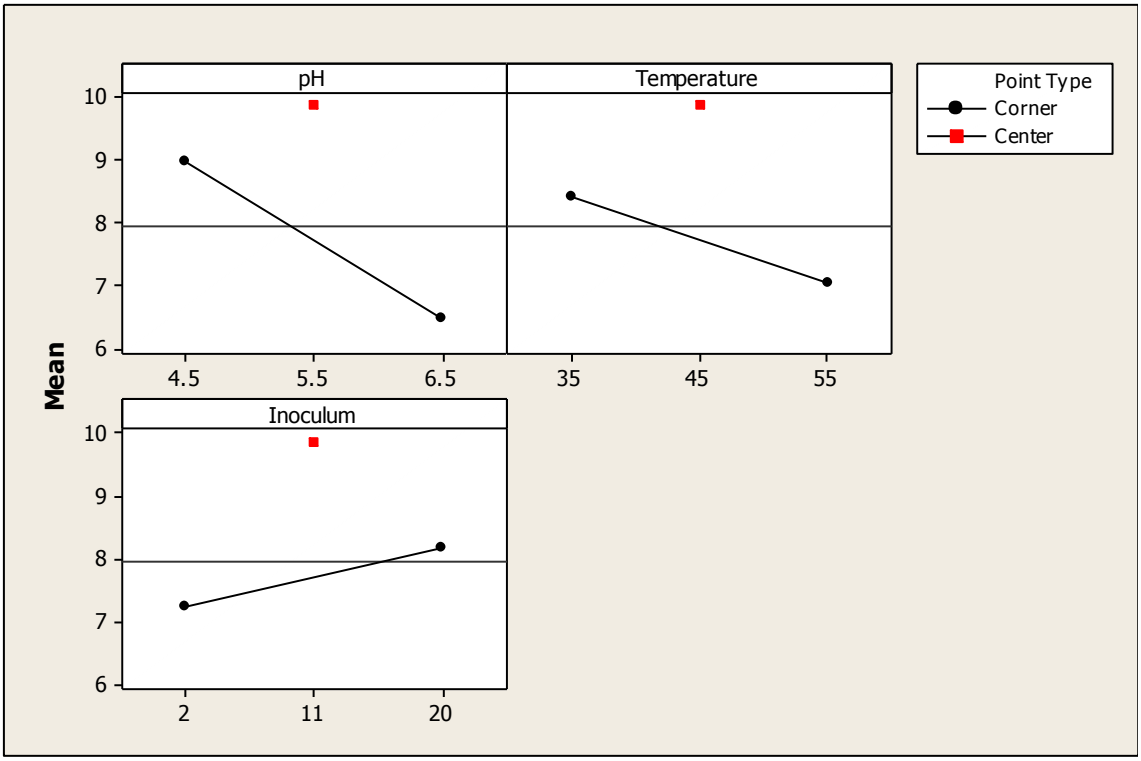

Figure 4.20: Main effects plot of hydrogen production from food waste mixed with POME as substrate (Production 2).

The effectiveness of factor-factor interactions are represented in Figure 4.21. This interaction effects plot was generated from ANOVA analysis as in Table 4.7. Based on that table, none of the interactions showed significant effect at $\alpha = 0.05$. Hence the resulting plots all having two lines running parallel to each other.

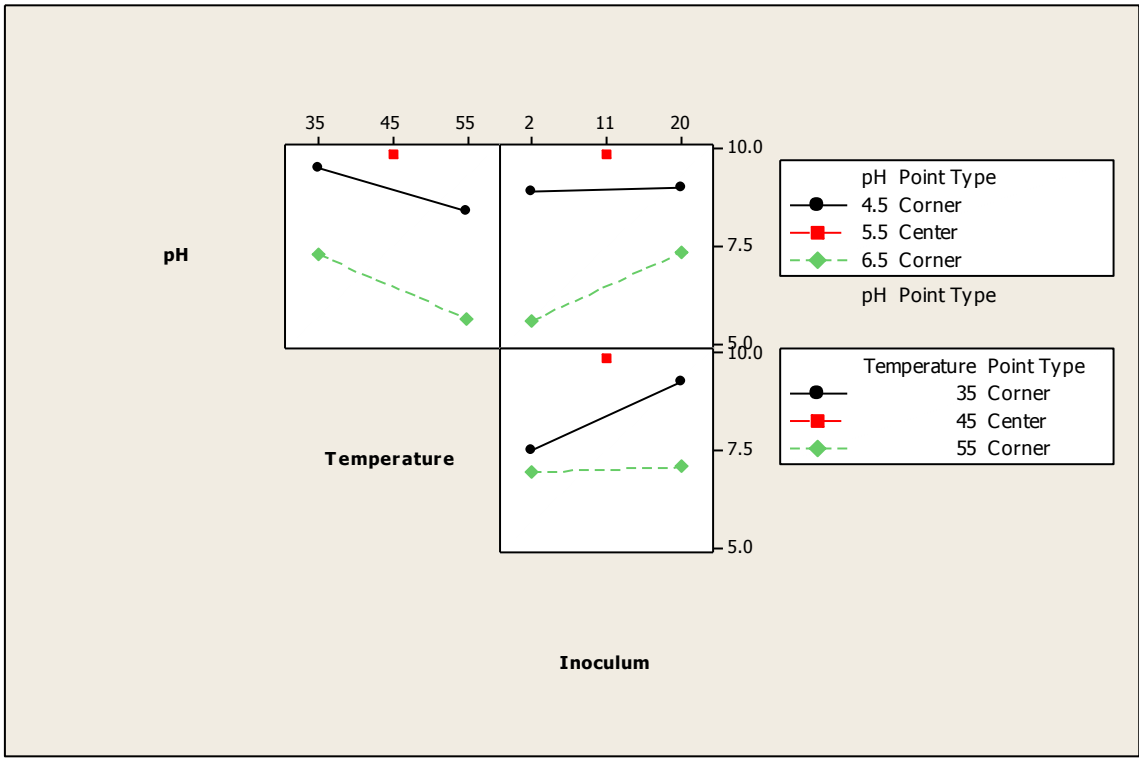

Figure 4.21: Interaction plot for hydrogen produced in Production 2

Figure 4.22 is the standardized residual plots for cumulative hydrogen production in Production 2. A normal distribution was suggested by experimental points being reasonably aligned. Most of the data points in the normal probability plot of the standardized residual all falls fairly close to the middle line (Gottipati and Mishra, 2010).

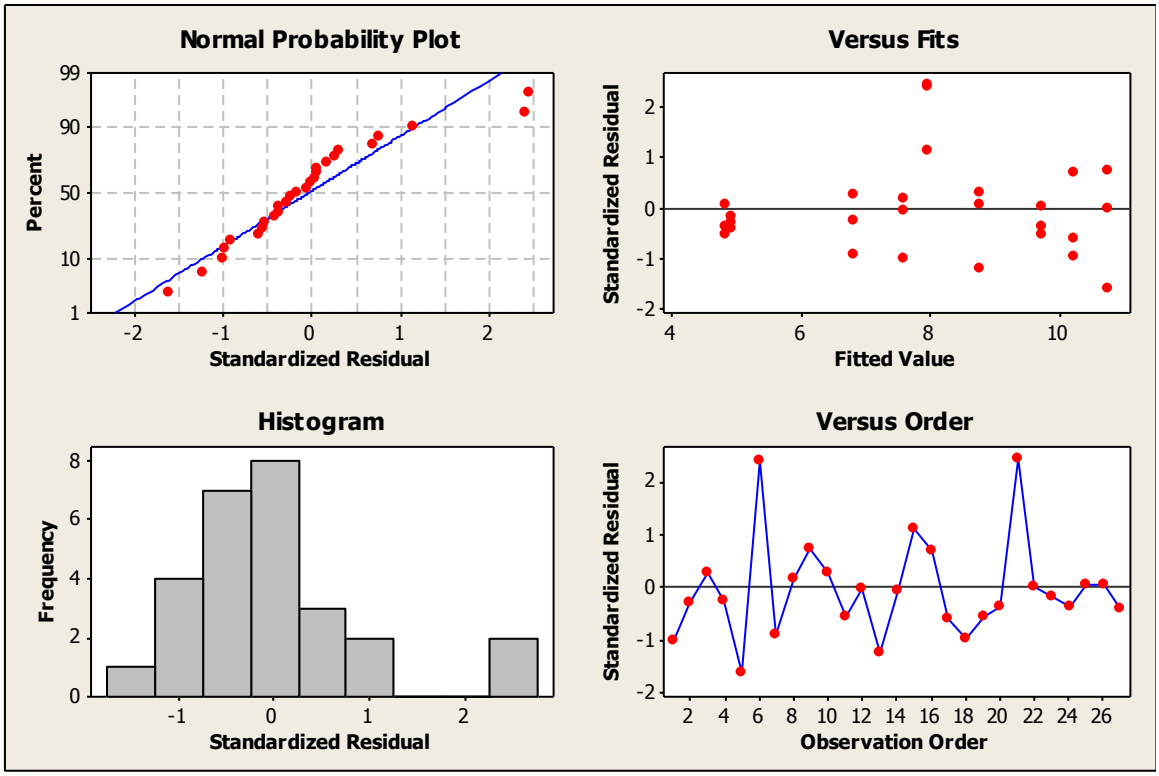

Figure 4.22: Residual plots for hydrogen production in Production 2

The normal plot of standardized effect compares the observed values of the variable to the observations expected for a normally distributed variable. Each value obtained is paired with its theoretical normal distribution forming a linear pattern. If the sample is from a normal distribution, then the observed value or scores fall more or less in a straight line; the blue line as in Figure 4.23.

In Figure 4.23, all the main factors *A*, *B* and *C* and also the 3 way interactions of *ABC* showed significance. Only the factor inoculum size, *C*, lies on the right side of the line. This means that it showed positive effect on the cumulative hydrogen gas production. The rest of the significance points lie on the left side of the line, indicating their negative effect on the response. The strength of influence of the factors are in ascending manner; *C* > *B* > *A* > *ABC*. This was concluded from the distance of the points from the line.

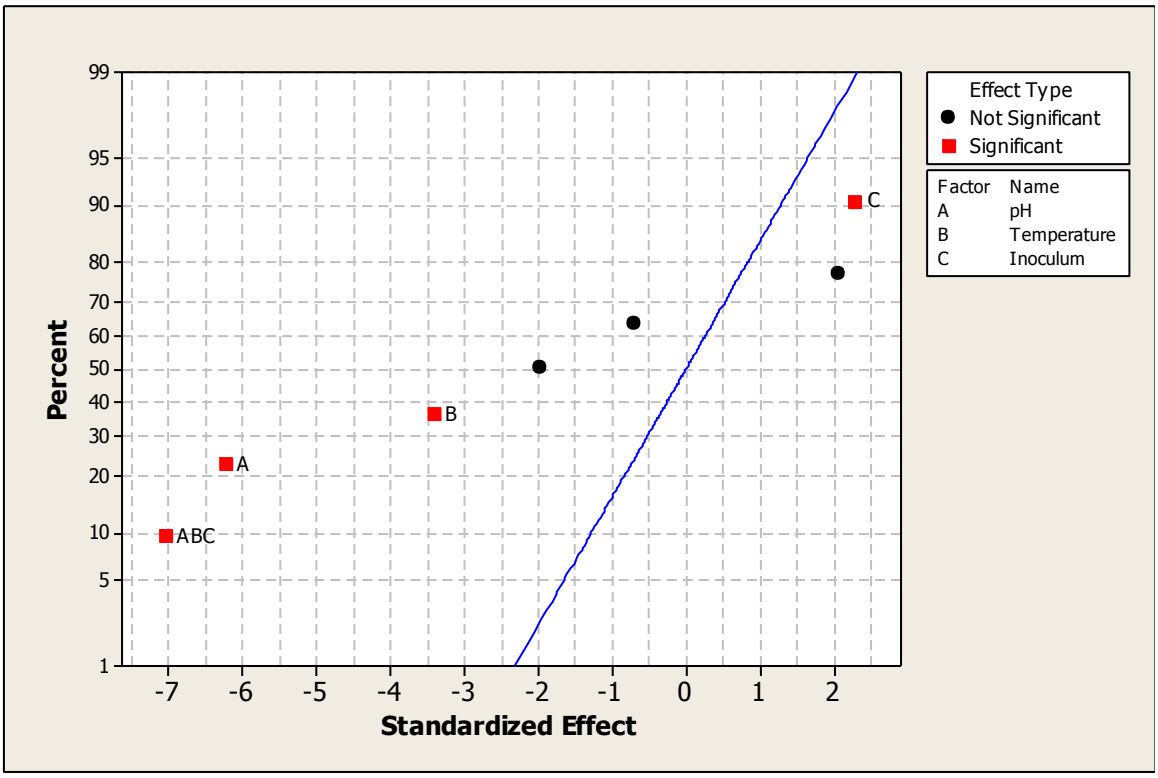

Figure 4.23: Normal plot of the standardized effects for Production 2

In Figure 4.24, 4.25 and 4.26, all these contour plots have circular contour that explains the insignificance of the interactions of *AB*, *BC* and *AC*. The arrows show the trajectory of optimization. In Figure 4.24, the optimization moved towards higher inoculum size and lower initial pH, when temperature was kept constant at 35°C.

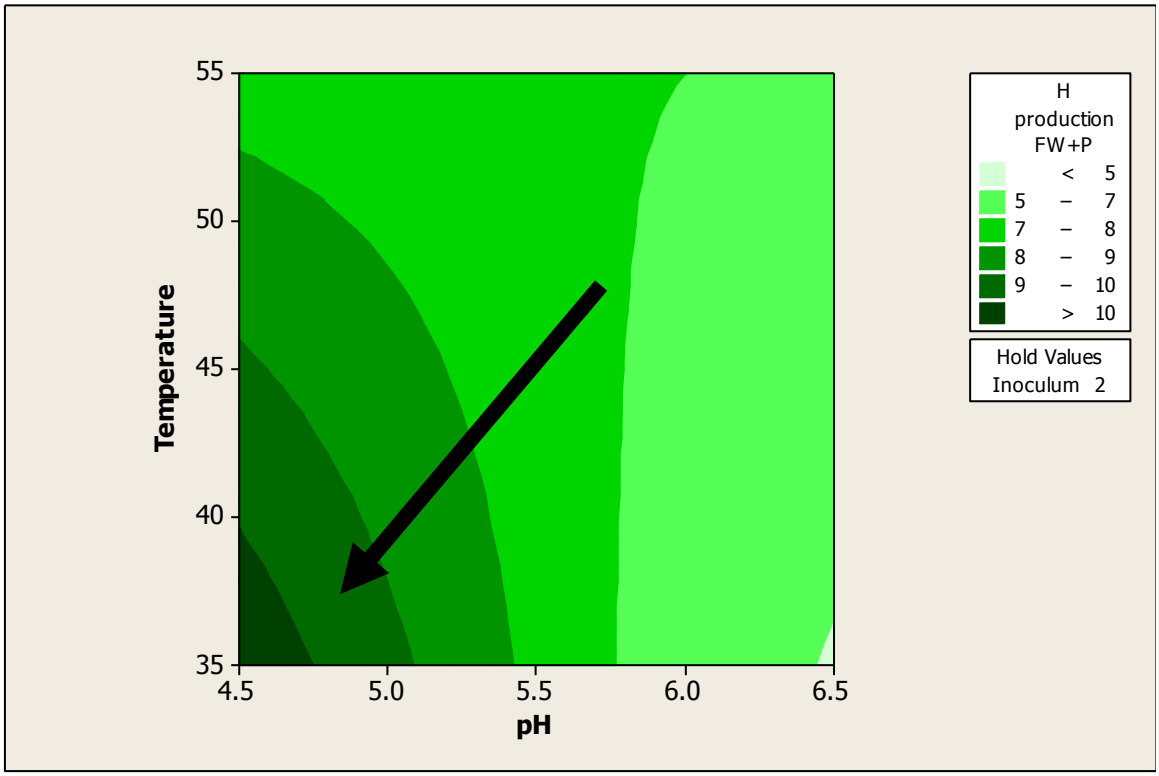

Figure 4.24: Contour plot of hydrogen production from food waste and POME (Production 2) versus temperature and initial pH

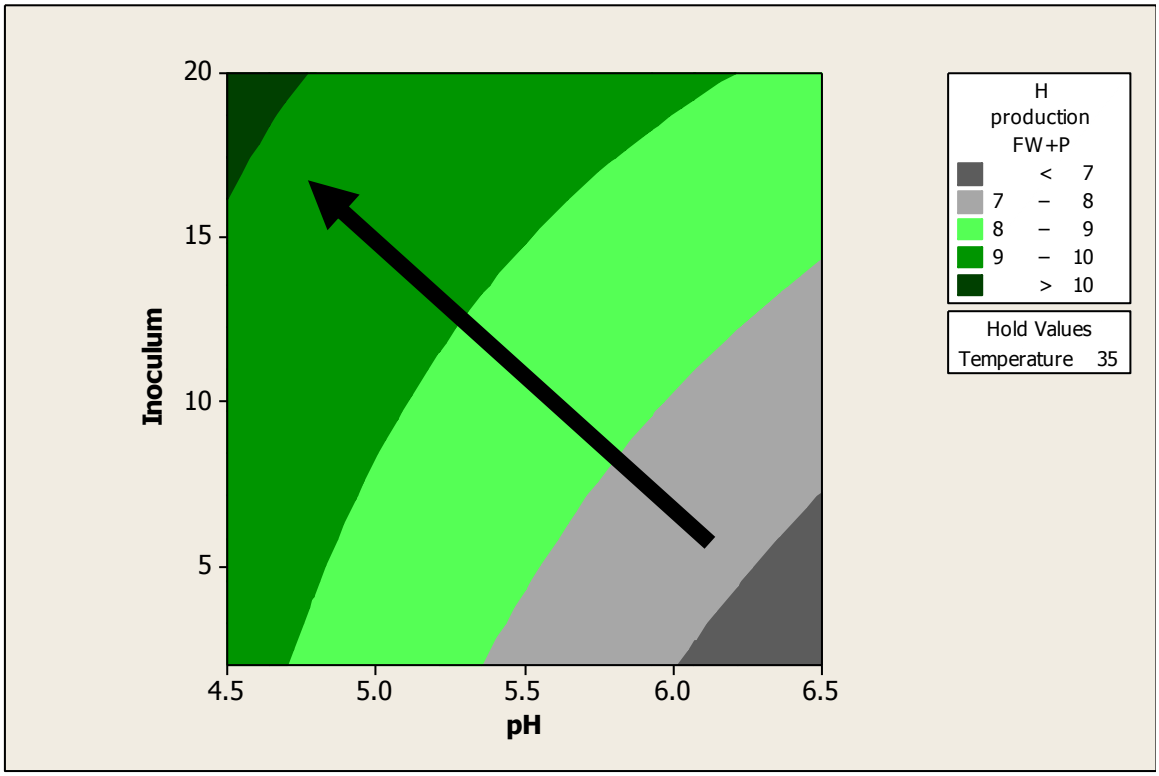

Figure 4.25: Contour plot of hydrogen production from food waste and POME (Production 2) versus inoculum size and initial pH

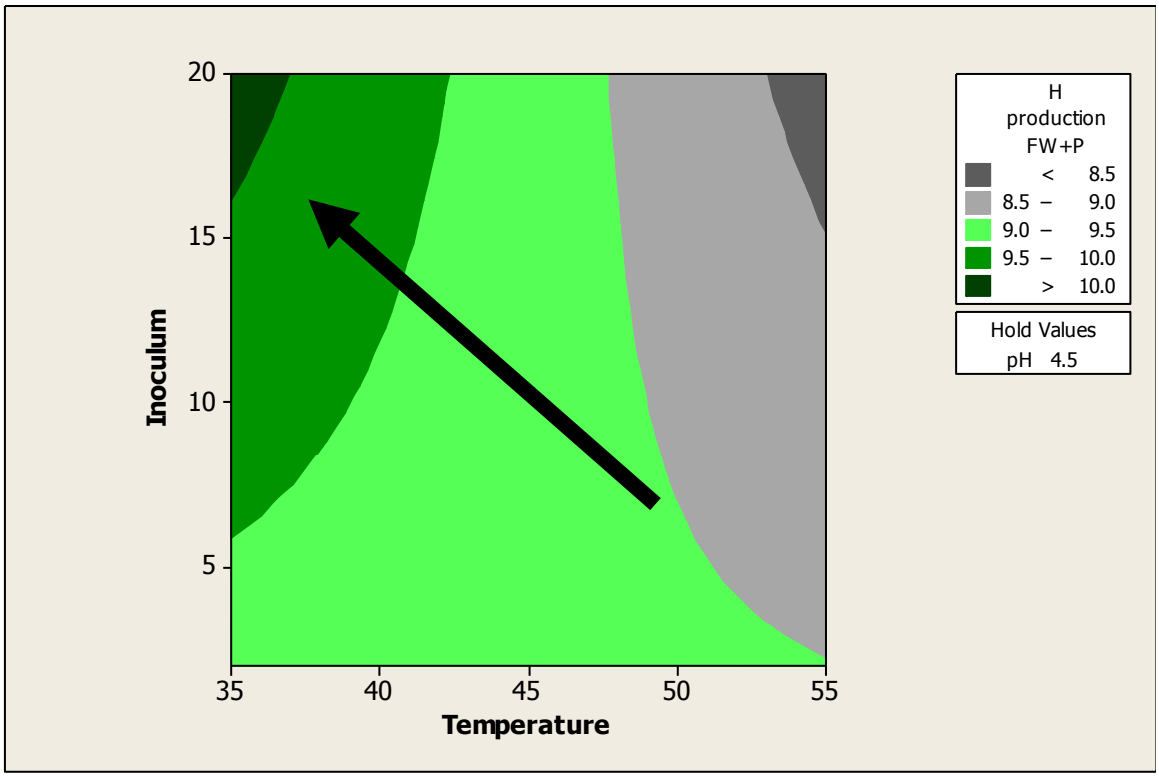

Figure 4.26: Contour plot of hydrogen production from food waste and POME (Production 2) versus inoculum size and temperature.

Figure 4.27 showed a cube plot to see the predicted responses based on a three way interactions of the factors *A*, *B* and *C*. For Production 2, the highest response would be at lowest initial pH (*A*), lowest temperature (*B*) and lowest inoculum size (*C*).

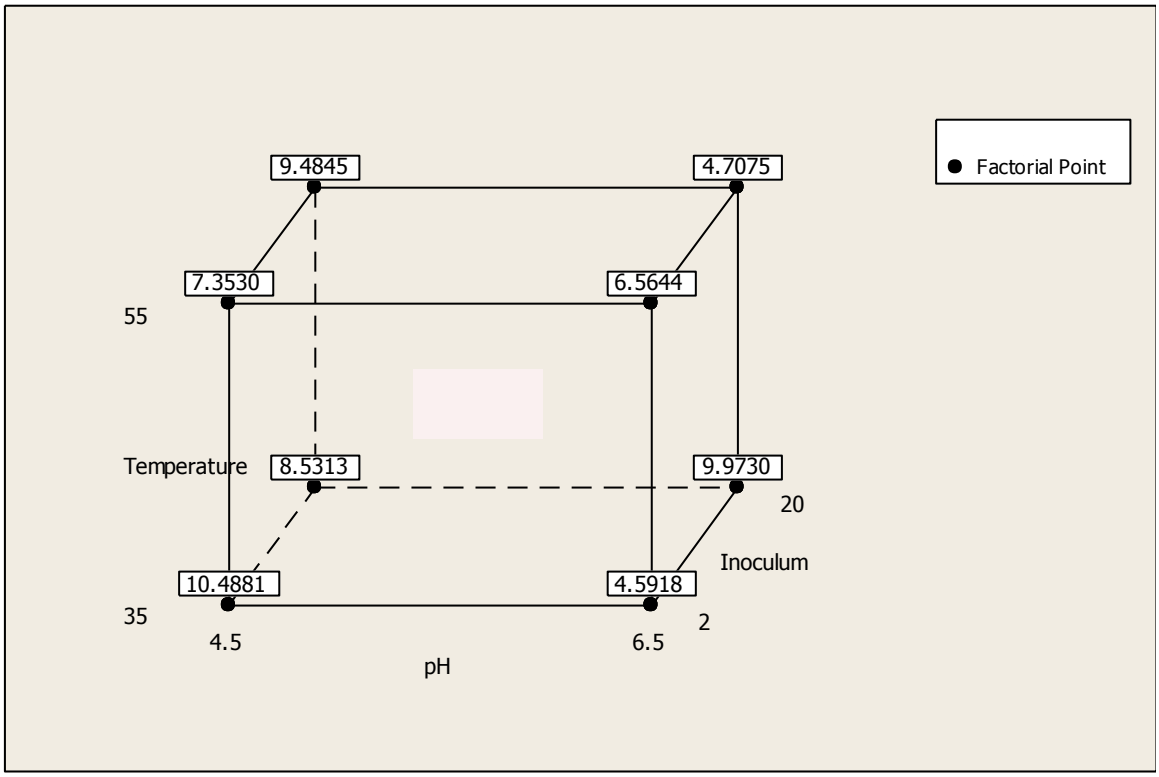

Figure 4.27: Cube plot for hydrogen production from substrate of food waste and POME (Production 2)

From the response optimizer function in Minitab® software, the maximum predicted hydrogen production of 10.73 ml can be obtained with initial pH of 4.5, inoculum size of 2% and temperature of 35°C. This also agreed with the cube plot (Figure 4.27). The composite desirability given by the Minitab software was 0.9319 which indicates that it is possible to obtain predicted results in 93 times out of 100 runs.

4.5 Verification Experiment at Optimized Condition

Figure 4.28 and 4.29 are overlaid contour plots for both cumulative hydrogen gas and COD removal. The white regions indicate the possible region for simultaneous optimization of the responses. In Figure 4.28, at temperature range 35 to 38°C and initial pH range 4.5 to 4.8, more than 8 ml of hydrogen can be produced with more than 65% COD removal for Production 1 when the inoculum size is hold at 20%. The possible region for simultaneous optimization of initial pH and inoculum size for Production 2 is shown in white area in Figure 4.29. When temperature is hold at 35°C with inoculum range 10 - 20% and pH range 4.5 - 6.5, more than 8.3 ml of hydrogen and 78% COD can be removed.

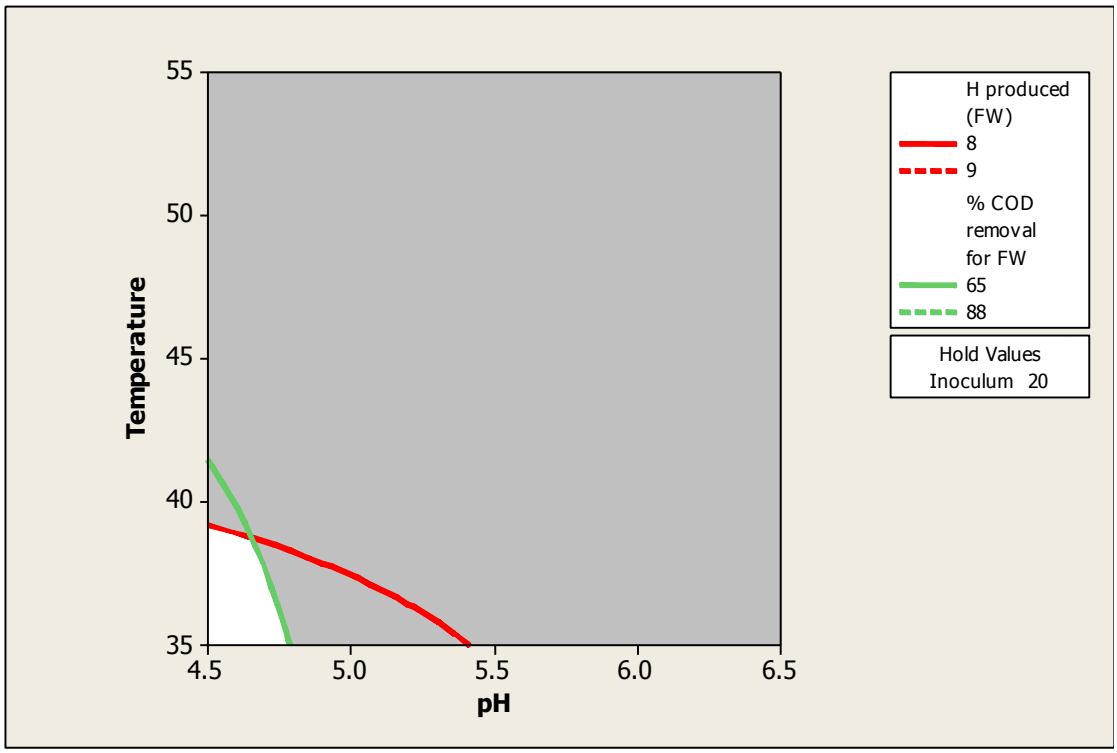

Figure 4.28: Overlaid contour plot for cumulative hydrogen production (ml) and COD removal (%) versus pH and temperature for Production 1.

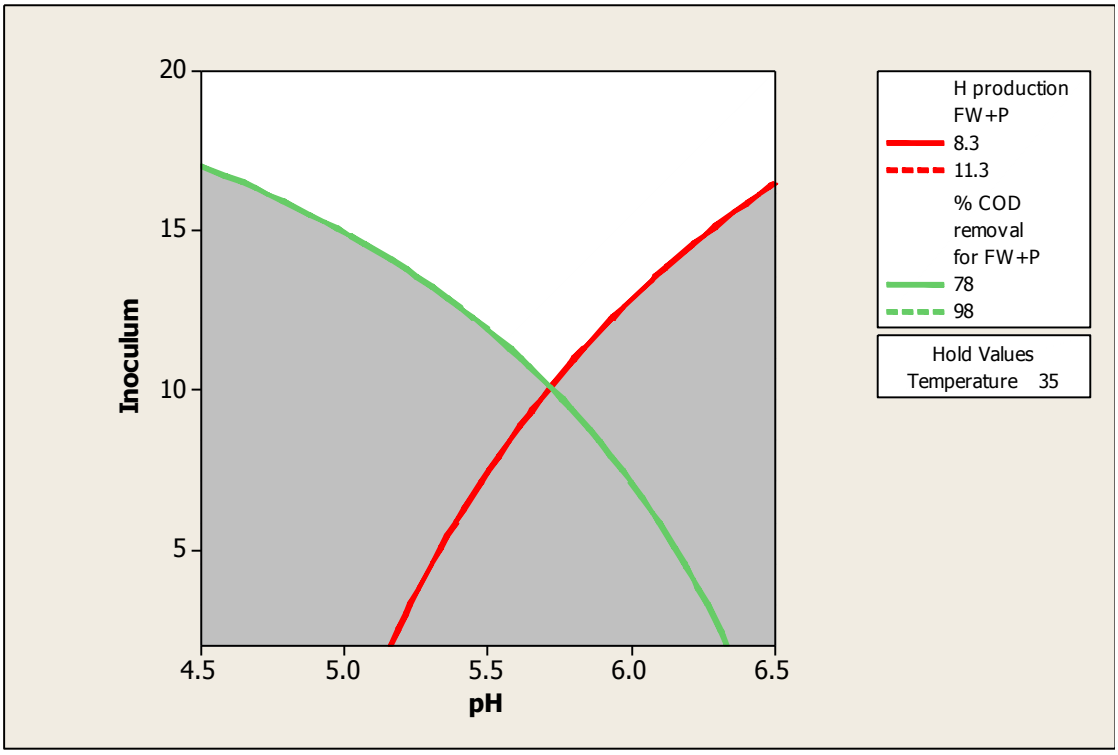

Figure 4.29: Overlaid contour plot for cumulative hydrogen production (ml) and COD removal (%) versus pH and inoculum for Production 2

Table 4.7 summarizes the expected cumulative hydrogen production as obtained from the response optimizer on Minitab® software and also the actual result obtained from verification experiment. At given optimum set of parameters, both Production 1 and 2 produced cumulative hydrogen slightly higher than expected value. The COD removal for both Production 1 and 2 however were lower than expected. It is possible that the increase in the value of one response may be achieved at the expense of the other response i.e. in this case hydrogen production and COD removal, respectively.

Table 4.7: Summary of results for verification experiment

	Optimum set of parameters			Expected		Observed	
Productions	Initial pH	Temperature (°C)	Inoculum size (% vol/vol)	Cumulative Hydrogen production	COD removal	Cumulative Hydrogen production	COD removal
Production 1 (food waste)	4.5	35	20	0.22 ml H_2/ ml substrate	68.79%	0.28 ml H_2/ ml substrate	41.73%
Production 2 (food waste with POME)	4.5	35	2	0.26 ml H_2/ ml substrate	81.62%	0.33 ml H_2/ ml substrate	75.59%

The food waste mixed with POME (Production 2) produced more hydrogen than food waste as the only substrate since POME itself contain a number of hydrogen producing microbes. Ismail *et al* (2009) has demonstrated how POME can be used as inoculum. Hence the addition of POME to microbe-rich sewage sludge will only enhance the hydrogen producing capability. Lay *et al* (2000) studied the feasibility of hydrogen production under mesophilic condition using mixed anaerobic bacteria. At high F/M ratio (0.4 g solid waste/ g biomass) the pre-treated digested sludge had SHPR of 43 ml hydrogen/ g VSS/ hour.

Hydrogen gas production occurs at acidogenic stage of anaerobic metabolism hence a slightly acidic pH is considered optimal (Vasquez and Varaldo, 2009). Normally the optimum pH for hydrogen production in dark fermentation process is within the range of 5.0 to 7.0, with the common optimum pH at 5.5 as shown in the study by Muralidhar *et al* (2001) and Van Ginkel *et al* (2001). In this study, pH 4.5 was the most suitable pH for the simultaneous production of hydrogen and COD reduction. A study by Fang *et al* (2006) showed that at low pH of 4.5, hydrogen production process is most effective.

In another study by Khanal *et al* (2004), the optimal initial pH was also found to be pH 4.5. At higher initial pH, rapid hydrogen production occurs alongside acid up to an inhibitory level which simultaneously depletes the buffering capacity. Bacteria could not adapt to this sudden change in environment and died. At lower initial pH, a longer lag period is exhibited for adaptation with the hydrogen gas produced at a moderate level. Ginkel *et al* (2001) explained that when the concentration of acid is high at high initial pH, the ionic strength of the solution will increase resulting in hydrogen production to switch to solvent production. The pattern of intermediate VFAs is different under variable pH conditions. The main products are butyrate and acetate and

hydrogen-producing butyrate-acetate pathway is favoured at pH 4.5 to 6.0 (Pakarinen *et al*, 2008).

Temperature has great influence on the activity of hydrogen producing bacteria. Different studies showed different optimum temperature but fermentative hydrogen production most commonly fell into the mesophilic range; around 37°C (Fang *et al*, 2007) which agreed well with the temperature obtained in the study. In the case of food waste, mesophilic temperatures seems to be more suitable for hydrogen production though there have been conflicting findings in literature. This may be due to the different type of inoculum and pretreatment method used and also the complex nature of food waste composition. Studies done by Li *et al* (2008) and Kim *et al* (2004) using batch system at 35°C, reported production of 196 ml hydrogen/ g VSS and 60 ml hydrogen/ g VSS, respectively.

The quantity of readily-degradable compounds and the operating conditions too can influence hydrogen production activity too. Okamoto *et al* (2000) reported results for hydrogen production in a batch reactor at 35°C using substrates from food wastes; rice, cabbage and meat. Highest hydrogen yield was obtained with rice (96 ml hydrogen/ g VSS) and lowest with meat (8 ml hydrogen/ g VSS). Since rice is more readily degradable compared to cabbage and complex protein like meat, it produced more hydrogen. The consumption of hydrogen gas to form ammonium using nitrogen from biodegradation of protein rich solid waste could also explain the low hydrogen gas yield when meat was used as a substrate (Lay *et al*, 2003).

5.0 CONCLUSIONS

The use of mixed culture for fermentative hydrogen production is more practical than a pure culture as this method of hydrogen gas production is simpler to operate and control. It also has more variety for source of substrate to be utilized. Heat pre-treatment method applied has successfully suppressed hydrogen consuming methanogens based on the absence of methane gas in GC analysis.

The operating conditions are indeed important to be considered when designing a system. In this study, temperature, inoculum size and initial pH definitely affected the microbial hydrogen production.

An anaerobic and facultative anaerobic system did not show much difference in terms of hydrogen yield. The possibility of using facultative anaerobes for hydrogen production will eliminate the need for gas sparging which in turn will reduce production cost.

Response Surface Methodology (RSM) applied in this study has proven to be effective in optimizing the system for microbial hydrogen production. It is feasible to use food waste with or without further supplementation by POME as a substrate for microbial hydrogen gas production. The system not only produces hydrogen as new source of energy but also reduce the COD of the waste.

For future works, the optimized conditions can be scaled-up and replicated. A batch system 3 litre stirred tank reactor should be sufficient as a first step to test the optimized conditions at a larger scale. However, attention should be given to the ratio of upscaling. The production of volatile fatty acids should also be studied since it can affect the microbial hydrogen production.

In the long run, the development of bacterial strains that produces mainly acetic and butyric acid with no lactic acids, propionic acids and alcohol through means of genetic engineering can further improve microbial hydrogen production. A combined photosynthetic and anaerobic bacterial system can also be applied to produce higher yield of microbial hydrogen since the photosynthetic bacteria can convert the by-products of anaerobic bacteria into hydrogen. Theoretically the system's microbial hydrogen production will increase four-fold, from 6 mol to 24 mol of hydrogen.

REFERENCES

APHA (1992). *Standard Methods for the Examination of Water and Wastewater* (18th ed.). Washington, DC: American Public Health Association.

Bradley, N. (2007). *The response surface methodology* (Master's thesis). Retrieved from https://www-test.iusb.edu/

Chen, W., Chen, S., Khanal, S. K., & Sung, S. (2006). Kinetic study of biological hydrogen production by anaerobic fermentation. *International Journal of Hydrogen Energy, 31*, 2170-2178.

Chittibabu, G., Nath, K., & Das, D. (2006). Feasibility studies on the fermentative hydrogen production by recombinant *Escherichia coli* BL-21. *Process Biochemistry, 41*, 682-688.

Chong, M., Sabaratnam, V., Shirai, Y., & Hassan, M. A. (2009). Biohydrogen production from biomass and industrial wastes by dark fermentation. *International Journal of Hydrogen Energy, 34*, 3277-3287.

Chu, C., Ebie, Y., Xu, K. Q., Li, Y., & Inamori, Y. (2010). Characterization of microbial community in the two-stage process for hydrogen and methane production from food waste. *International Journal of Hydrogen Energy, 35*, 8253-8261.

Chu, C., Li, Y., Xu, K. Q., Ebie, Y., Inamori, Y., & Kong, H. N. (2008). A pH and temperature phased two-stage process for hydrogen and methane production from food waste. *International Journal of Hydrogen Energy, 33*, 4739-4746.

Clark, I., C., Zhang, R., H., & Upadhyaya, S. K. (2012). The effect of low pressure and mixing on biological hydrogen production via anaerobic fermentation. *International Journal of Hydrogen Energy, 37*, 11504-11513.

Dagoumas, A. S., Papagiannis, G. K., & Dokopoulos, P. S. (2006). An economic assessment of the Kyoto Protocol application. *Energy Policy, 34*(1), 26-39.

Doelle, H. W. (1994). *Microbial process development*. Singapore: World Scientific Publishing.

Elbeshbishy, E., Hafez, H., Dhar, B. R., & Nakhla, G. (2011). Single and combined effect of various pretreatment methods for biohydrogen production from food waste. *International Journal of Hydrogen Energy,* 1-9.

Fang, H. H. P., Li, C. L., & Zhang, T. (2006). Acidophilic biohydrogen production from rice slurry. *International Journal of Hydrogen Energy, 31*, 683-692.

Fang, H. H. P., & Li, C. L. (2007). Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. *Environmental Science Technology, 37*, 1-39.

Ginkel, S. V., Sung, S. W., & Lay, J. J. (2001). Biohydrogen production as a function of pH and substrate concentration. *Journal of Environmental Science and Technology*, *35*, 4726-4730.

Guo, X. M., Trably, E., Latrille, E., Carrere H., & Steyer, J. (2010). Hydrogen production from agricultural waste by dark fermentation: a review. *International Journal of Hydrogen Energy, 35*, 10660-10673.

Han, S., & Shin, H. (2004). Biohydrogen production by anaerobic fermentation of food waste. *International Journal of Hydrogen Energy, 29*, 569-577.

Hughes, S., Qureshi, N., Maddox, I. S., Cotta, M. A. (2005). Energy-efficient recovery of butanol from model solutions and fermentation broth by adsorption. *Bioprocess and Biosystem Engineering, 27* (4), 215-222.

Ibrahim, M. F., Abd-Aziz, S., Abdul Razak, M.N., Phang, L.Y., and Hassan, M.A. (2012). Oil empty fruit bunch as alternative substrate for acetone-butanol-ethanol production by Clostridium butyricum EB6. *Applied Biochemical and Biotechnology, 166*, 1615-1625.

Ismail, F., Abdul Rahman, N., Abd Aziz, S., Ling, C. M., & Hassan, M. A. (2009). Statistical optimization of biohydrogen production using food waste under thermophilic conditions *The Open Renewable Energy Journal, 2*, 124-131.

Jo, J. H., Lee, D. S., Park, D., & Park, J. M. (2008). Biological hydrogen production by immobilized cells of *Clostridium tyrobutyricum* JM1 isolated from a food waste treatment process. *Bioresource Technology, 99*, 6666-6672.

Kapdan, I. K., & Kargi, F. (2006). Bio-hydrogen production from waste materials. *Enzyme and Microbial Technology, 38*, 569-582.

Kawagoshi, Y., Hino, N., Fujimoto, A., Nakao, M., Fujita, Y., & Sugimura, S. (2005). Effect of inoculum conditioning on hydrogen fermentation and pH effect on bacterial community relevant to hydrogen production. *Journal of Biosciences and Bioengineering, 100*, 524-530.

Khanal, S. K., Chen, W., Li, L., & Sung, S. (2004). Biological hydrogen production: effects of pH and intermediate products. *International Journal of Hydrogen Energy, 29*, 1123-1131.

Kim, S., Han, S., & Shin, H. (2004). Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge. *International Journal of Hydrogen Energy, 29*, 1607-1616.

Kim, D., Wu, J., Jeong, K., Kim, M., & Shin, H. (2011). Natural inducement of hydrogen from food waste by temperature control. *International Journal of Hydrogen Energy, 36*, 10666-10673.

Kumar, N., & Das, D. (2000). Enhancement of hydrogen production by *Enterobacter cloacae* IIT-BT 08. *Process Biochemistry, 35*, 589-593.

Lay, J., Li, Y., & Noike, T. (1998). Mathematical model for methane production from landfill bioreactor. *Journal of environmental Engineering*, 730-736.

Lee, Y., & Chung, J. (2010). Bioproduction of hydrogen from food waste by pilot-scale combined hydrogen/methane fermentation. *International Journal of Hydrogen Energy, 35*, 11746-11755.

Levin, D. B., Islam, R., Cicek, N., & Sparling, R. (2006). Hydrogen production by *Clostridium thermocellum* 27405 for cellulosic biomass substrates. *International Journal of Hydrogen Energy, 31*, 1496-1503.

Liu, D., Min, B., & Angelidaki, I. (2008). Biohydrogen production from household solid waste (HSW) at extreme-thermophilic temperature (70°C) – influence of pH and acetate concentration. *International Journal of Hydrogen Energy, 33*, 6985-6992.

Luo, G., Zou, Z., Zhou, Q., & Wang, J. (2010). Fermentative hydrogen production from cassava stillage by mixed anaerobic microflora: Effects of temperature and pH. *Applied energy, 87*, 3710-3717.

Manni G., & Caron F. (1995). Calibration and determination of volatile fatty acids in waste leachates by gas chromatography. *Journal of Chromatography A, 690*, 237.

Ming, L., Youcai, Z., Qiang, G., Xiaoqing, Q., & Dongjie, N. (2008). Bio-hydrogen production from food waste and sewage sludge in the presence of aged refuse excavated from refuse landfill. *Renewable Energy, 33*, 2573-2579.

Mohammadi, P., Shaliza Ibrahim, Mohamad Suffian Mohamad Annuar, & Law, S. (2011). Effects of different pretreatment methods on anaerobic mixed microflora for hydrogen production and COD reduction from palm oil mill effluent. *Journal of Cleaner Production, 19*, 1654-1658.

Moreno-Davila, I. M. M., Rios-Gonzalez, L. J., Garza-Gracia, Y., Garza, J. A. R., & Rodriguez-Martinez, J. (2011). Biohydrogen production from dairy processing wastewater by anaerobic biofilm reactors. *African Journal of Biotechnology, 10*(27), 5320-5326.

Muralidhar, R. V., Chirumamila, R. R., Marchant, R., & Nigam, P. (2001). A response surface approach for the comparison of lipase production by *Candida cylindracea* using two different carbon sources. *Biochemical Engineering Journal*, *9*, 17-23.

Nath, K., Kumar, A., & Das, D. (2005). Hydrogen production by *Rhodobacter sphaeroides* strain O.U.001 using spent media of *Enterobacter cloacae* strain DM11. *Applied Microbial and Cell Physiology, 68*, 533-541.

Nath, K., & Das, D. (2011). Modelling and optimization of fermentative hydrogen production. *Bioresource Technology, 102*, 8569-8581.

Okamoto, M., Noike, T., Miyahara, T., & Mizuno, O. (2000). Biological hydrogen potential of materials characteristics of the organic fraction of municipal solid wastes. *Water Sciencs and Technology, 41*, 25-32.

Pakarinen, O., Lehtomaki, A., & Rintala, J. (2008). Batch dark fermentative hydrogen production from grass silage: the effect of inoculum, pH, temperature and VS ratio. *International Journal of Hydrogen Energy*, *33*(2), 594-601.

Pan, J., Zhang, R., El-Mashad, H. M., 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.

Porporato, P. E., Dhup, S., Dadhich, R. K., Copetti, T., Sonveaux, P. (2011). Anticancer targets in the glycolytic metabolism of tumors: a comprehensive review. *Frontiers in Pharmacology.* Retrieved from http://journal.frontiersin.org/Journal/10.3389/fphar.2011.00049/full

Shin, H., Youn, J., & Kim, S. (2004). Hydrogen production from food waste in anaerobic mesophilic and thermophilic acidogenesis. *International Journal of Hydrogen Energy, 29*, 1355-1363.

Sinha, P., & Pandey, A. (2011). An evaluative report and challenges for fermentative biohydrogen production. *International Journal of Hydrogen Energy, 36*, 7460-7478.

Sen, D., & Das, D. (2005). Multiple parameter optimization for the maximazation of hydrogen production by *Enterobacter cloacae* DM11. *Journal of Scientific and Industrial Research*, *64*, 984-990.

Siedlecka, E. M., Kumirska, J., Ossowski, T., Glamowski, P., Golebiowski, M., Gajdus, J., Kaczynski, Z., & Stepnowski, P. (2008). Determination of volatile fatty acids in environmental aqueous samples. *Polish Journal of Environmental Studies, 17*, 351-356.

Song, C. (2003, Sep). *Overview of hydrogen production options for hydrogen energy development, fuel-cell fuel processing and mitigation of CO_2 emissions*. Paper presented at the International Pittsburgh Coal Conference.

Sreela-or, C., Imai, T., Plangklang, P., & Reungsang, A. (2011). Optimization of key factors affecting hydrogen production from food waste by anaerobic mixed cultures. *International Journal of Hydrogen Energy,* 1-14.

Vasquez, I. V., & Varaldo, H. M. P. (2009). Hydrogen production by fermentative consortia. *Journal of Renewable and Sustainabale Energy*, *13*, 1000-1013.

Villanueva, A. (2007). Recycling, incineration or landfilling? A review of existing life cycle assessments. *Waste Management, 27*, 29-46.

Wang, J., & Wan, W. (2008). Comparison of different pretreatment methods for enriching hydrogen-producing bacteria from digested sludge. *International Journal of Hydrogen Energy, 33*, 2934-2941.

Wang, J., & Wan, W. (2009). Factors influencing fermentative hydrogen production: a review. *International Journal of Hydrogen Energy, 34*, 799-811.

Wang, J., & Wan, W. (2009). Experimental design methods for fermentative hydrogen production: a review. *International Journal of Hydrogen Energy, 34*, 235-244.

Ward, A. J., Hobbs, P. J., Holliman, P. J., Jones, D. L. (2008). Optimisation of the anaerobic digestion of agricultural resources. *Bioresource Technology, 99* (17), 7928-7940.

World Energy Outlook. (2006). France: International Energy Agency.

Zadariana Jamil, Mohamad Suffian Mohamad Annuar, Shaliza Ibrahim, & Vikineswary Sabaratnam. (2009). Optimizaton of phototrophic hydrogen production by *Rhodopseudomonas palustris* PBUM001 via statistical experimental design. *International Journal of Hydrogen Energy*, *34*, 7502-7512.

Zheng, X., & Yu, H. (2005).Inhibitory effects of butyrate on biological hydrogen production with mixed anaerobic cultures. *Journal of Environmental Management, 74*, 65-70.

Zhu, H., Parker, W., Basnar, R., Proracki, A., Falletta, P., Beland, M., & Seto, P. (2009). Buffer requirements for enhanced hydrogen production in acidogenic digestion of food wastes. *Bioresource Technology*, *100*, 5097-5102.