PRELIMINARY STUDY ON BIOHYDROGEN PRODUCTION FROM OLEOCHEMICAL WASTE

DAYANG RAFIKA ATIQAH BINTI ABANG OTHMAN

UNIVERSITI MALAYSIA PAHANG

UNIVERSITI MALAYSIA PAHANG

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Alamat Tetap: No 154,Jalan Kpg Hilir, 95000 Sri Aman, Sarawak	**ROZAIMI ABU SAMAH** Nama Penyelia
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Supervisor : Rozaimi bin Abu Samah
Date : ....

PRELIMINARY STUDY ON BIOHYDROGEN PRODUCTION FROM OLEOCHEMICAL WASTE

DAYANG RAFIKA ATIQAH BINTI ABANG OTHMAN

A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

APRIL 2009

I declare that this thesis entitled ‘Preliminary Study on Biohydrogen Production from Oleochemical Waste’ is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

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Special Dedication to my family members,
my companion, my fellow colleague
and all faculty members

For all your care, support and believe in me.

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ABSTRACT

Hydrogen has become centre of global attention with its potential of being alternative fuel. It has indeed a favorable choice in dealing with fossil fuel depletion and high demand problem. Not only it produces a clean future fuel since it only produces water upon combustion; it also provides a solution for abundant waste. This study focused on the anaerobic fermentation, one of the biological methods available with the use of oleochemical industry waste. The objective of this study was to determine the hydrogen producing strain microorganisms that were capable in utilizing oleochemical waste and hence producing hydrogen. Mixed culture obtained from treated and untreated oleochemical waste was used in this study. Three types of medium used in this study were consisted from TYA medium, autoclaved untreated oleochemical waste and autoclaved treated oleochemical waste. Whereas the trials carried out in this study were based on the several combinations of different medium and inoculums. Results obtained in trials conducted were not conclusive since the amount of gas released upon fermentation was very minimal. Although the hydrogen detection system 3 showed some promising result in Trial 11, repetitions were not able to confirm the result obtained due to some technical problems encountered. As a conclusion, this study managed to design three detection systems that would show conclusive results with presence of substantial amount of hydrogen. Thus, the contributing factors of which no detection of hydrogen and the recommendation has been discussed.

ABSTRAK

Umum kian mengenali potensi hidrogen selaku pengganti bahan bakar. Sememangnya hidrogen merupakan pilihan yang terbaik dalam mengatasi masalah kekurangan bahan bakar fosil dan permintaan yang tinggi. Hidrogen merupakan alternatif yang mesra alam kerana ianya bukan sahaja menghasilkan air apabila dibakar malahan ianya turut menjadi jalan penyelesaian terhadap bahan buangan yang kian bertambah. Kajian ini memberi fokus terhadap fermentasi secara anaerobik yang merupakan salah satu kaedah biologi yang boleh dilaksanakan dengan penggunaan bahan buangan oleokimia. Kajian-kajian yang terdahulu telah menunjukkan bahawa mikroorganisma anaerobik merupakan penghasil gas hidrogen yang terbaik. Kajian ini bertujuan untuk mengenalpasti jenis mikroorganisma penghasil gas hidrogen yang berkebolehan untuk hidup dalam bahan buangan oleokimia sekaligus menghasilkan hidrogen. Mikroflora campuran yang diperolehi dari bahan buangan oleokimia yang telah dirawat dan belum dirawat digunakan sebagai inokulum. Fermentasi dijalankan di dalam kelalang bulat yang disambungkan pada sistem pengesanan hidrogen dengan menggunakan tiga jenis media yang berbeza iaitu media TYA, bahan buangan oleokimia yang belum dirawat dan telah disteril dan bahan buangan oleokimia yang telah dirawat dan disteril. Penghasilan hidrogen dari fermentasi dibiarkan selama 48 jam dalam dua suhu berbeza. Namun begitu, sistem pengesanan hidrogen tidak memberikan hasil dapatan yang meyakinkan. Dengan itu, faktor penyebab dan penambahbaikan telah dibincangkan.

TABLE OF CONTENTS

CHAPTER	ITEM	PAGE
	TITLE PAGE	**iv**
	DECLARATION	**v**
	DEDICATION	**vi**
	ACKNOWLEDGEMENT	**vii**
	ABSTRACT	**viii**
	ABSTRAK	**ix**
	TABLE OF CONTENTS	**x**
	LIST OF FIGURES	**xiii**
	LIST OF TABLES	**xiv**
	LIST OF SYMBOLS / ABBREVIATIONS	**xv**
	LIST OF APPENDICES	**xvi**
1	**INTRODUCTION**	
	1.1 Background of Study	1
	1.2 Problem Statement	3
	1.3 Objective	4
	1.4 Scopes of Study	4
2	**LITERATURE REVIEW**	
	2.1 Introduction	5
	2.1.1 Hydrogen	5
	2.1.2 Pathways in Producing Hydrogen	7

		2.1.3 Comparison of Physical and Biological Pathways	8
	2.2	Oleochemical	11
		2.2.1 Applications of Oleochemical	11
	2.3	Substrates	12
		2.3.1 Types of Substrates	12
	2.4	Microorganism	15
		2.4.1 Hydrogen Producing Strain Microorganism	15
		2.4.2 Major Enzymes	16
		2.4.2.1 Reversible or Classical Hydrogenase	17
		2.4.2.2 Membrane Bound Uptake Hydrogenase	18
		2.4.2.3 Nitrogenase Enzyme	18
	2.5	Barriers	19
		2.5.1 Barriers in Producing Hydrogen Biologically	19
3	**METHODOLOGY**		
	3.1	Introduction	24
	3.2	Materials and Method	25
		3.2.1 Microorganism and Culture Conditions	25
		3.2.2 Medium Preparation	26
		3.2.3 Hydrogen Fermentation	27
		3.2.4 Hydrogen Detection System	27
	3.3	Optical Density of Broth Culture	30
	3.4	Storage of Microorganism	30
	3.5	Summary of Methodology	32
4	**RESULTS AND DISCUSSION**		
	4.1	Introduction	33
	4.2	Result and Discussion	34
		4.2.1 Result on Hydrogen Detection System	34
		4.2.1.1 Discussion on Hydrogen Detection System	36

	4.2.2 Result and Discussion on Optical Density Measurement	38
	4.2.3 Result and Discussion on Broth Colour	39
5	**CONCLUSION AND RECOMMENDATION**	
	5.1 Conclusion	43
	5.2 Recommendation	44
	5.2.1 Usage of Gas Chromatography	44
	5.2.2 Usage of Anaerobic Jar/Chamber	44
	5.2.3 Adjustment on the Fermenter Flask	45
	5.2.4 Minimize the Length of Tubing	47
	5.2.5 Repetition of trials	47
	5.2.6 Usage of Various Single Culture	48
	REFERENCES	**49**
	APPENDIX A	**52**
	APPENDIX B	**54**

LIST OF FIGURES

FIGURE	TITLE	PAGE
2.1	Chronology of Fuel Usage by Mankind	6
2.2	Hydrogen Production Routes from Biomass	7
2.3	Photo-Fermentation Process	9
2.4	Two-Stage Fermentation Process	10
2.5	Biocatalyzed Electrolysis	10
2.6	Mechanism of Hydrogenase and Nitrogenase Enzymes	17
2.7	Nitrogenase Catalyzed Hydrogen Formation	19
3.1	Hydrogen Detection System 1	28
3.2	Hydrogen Detection System 2	28
3.3	Colour Changes in pH Indicator	29
3.4	Hydrogen Detection System 3	30
4.1	Gas Collected in Trial 11	38
4.2	Trial 7 Broth Culture	40
4.3	Trial 8 Broth Culture	41
4.4	Trial 9 Broth Culture	41
4.5	Trial 10-14 Broth Culture	42
5.1	Fermenter Flask	46
5.2	Glass Connector	47

LIST OF TABLES

TABLE	TITLE	PAGE
2.1	Application of Oleochemical Based Product	11
2.2	Substrates Used and Biologically Producing Microorganism	14
2.3	Hydrogen Producing Microorganism	15
2.4	Advantages and Disadvantages of Different Types of Hydrogen Producing Microorganism	19
2.5	Barriers and Solution in Producing Hydrogen Biologically	21
3.1	Combination of Inoculums and Medium	25
4.1	Observation on Biohydrogen Detection System	34
4.2	Optical Density Measurement at 660 nm	39

LIST OF SYMBOLS/ABBREVIATIONS

rpm	-	rotation per minute
TYA	-	trytone-yeast-ammonia
K-solubilizer	-	kalium solubilizer
GC	-	gas chromatography
nm	-	nanometer
M	-	molar
mg	-	milligram
g	-	gram
h	-	hour
mg/L	-	milligram per liter
min	-	minutes
mL	-	milliliter
L	-	liter
%	-	percentage
°C	-	degree Celsius
µm	-	micrometer

LIST OF APPENDICES

APPENDIX	TITLE	PAGE
A.1	UV-Visible Single Beam Spectrophotometer (Model U-1800)	52
A.2	Shaking Water Bath (Model BS-21)	52
A.3	Double Stack Shaking Incubator Infors	53
A.4	Laminar Air Flow Cabinet (Model AHC-4A1)	53
B.1	Trial 10 Broth Culture	54
B.2	Trial 11 Broth Culture	54
B.3	Trial 12 Broth Culture	55
B.4	Trial 13 Broth Culture	55
B.5	Trial 14 Broth Culture	56

CHAPTER 1

INTRODUCTION

1.1 Background

Fossil fuel being one of the major contributors to global prosperity has indeed overly consumed over these years. Being an unsustainable fuel, it became harder to meet the global demand. Not only that, it is also one of the significant causes of global warming and air pollution. It is based on these reasons that the idea of creating and finding alternative source of fuel came about. Hydrogen, being a highly priced commodity to the industry has become favourite energy to be regarded as an alternative fuel (Sivaramakrishna *et al.*, 2008). Hydrogen produces a clean by-product which is water upon combustion thus it does not pose a threat to the environment. Apart from that, it does not contribute to green house effect, acid rain, ozone depletion and global warming.

Hydrogen can be produced in numerous pathways and that includes electrochemical processes, thermochemical processes, photochemical processes, photocatalytic processes, or photoelectrochemical processes (Han *et al.*, 2004). However there are two major pathways of producing hydrogen that has been the focus of various researches. They are process through photosynthetic fermentation and anaerobic fermentation. Producing hydrogen through anaerobic process is preferable in terms of cost and feasibility.

Biologically produced hydrogen results to a win-win solution. Not only being a clean fuel, it also solves the problem of industrial wastes. By using these industrial wastes as substrate for producing hydrogen, the wastes being dumped and treated will be reduced into something more beneficial. Use of industrial wastewater as substrate does not only help in treating the waste but also contribute to renewable extraction of clean gas (Sivaramakrishna *et al.*, 2008). Oleochemical industrial waste is one of the potential wastes that would likely to become substrate in producing hydrogen. It is abundant and rich in glycerols making it favourable to become the subject of this study.

In order to biologically produced hydrogen, a suitable substrate must be used. Most of the hydrogen producing microorganism feed on substrate such as glucose in order to function properly. The high cost of suitable feedstock that can be the substrate for hydrogen production lead to the usage of renewable biomass as feedstock. According to a study by Meher and Das (2008), they concluded that industrial waste has the potential to become a significant source of renewable hydrogen. However processes involved in using the low cost waste biomass require a much more expensive and complicated procedure compared to the derivation of hydrogen from natural gas. Even so, biomass' potential to provide a cleaner and safer environment is much more at stake for years to come. Not only that, waste biomass is rather in abundant amount that it can provide a cost effective way to boost the economy if it can be converted into something that has such a high value to the industry. Although using biomass as substrate in producing hydrogen is a breakthrough in technology today, its low hydrogen conversion efficiency and low

yield of hydrogen is at question (Das *et al.*, 2001). However, these can be overcome with the co-digestion use of microbial consortia which can increase the yield apart than improving reactor design and operating parameters.

1.2 Problem Statement

The need and demand for fuel has been sky rocketing for the past years as a result to increment price of world's crude oil. Fossil fuel that has been the major contributor to global economy is unsustainable but yet the demand for the energy is increasing year by year. Even so, fossil fuel is one of the major pollutants that endanger Mother Nature. Although it poses many threats to environment it is still remain an important element to drive the economy and create industry. With such limited stock of crude oil along with a high demand, the urge to find some other renewable energy come about. There are some suggestion and alternative solution to deal with the spiking fuel price. As the government suggested, hybrid cars save more on fuel. The problem with hybrid cars is the price per unit.

According to an article in Berita Harian dated 2nd August 2008, a Honda hybrid with engine capacity of 1.3 litres cost around RM 160,000-RM 170,000 compared to conventional car of the same capacity which only cost around RM110, 000-RM 120,000. It is rather obvious that the price of hybrid cars does not help in curbing the fuel price matter. Not only that, the hybrid cars still posses a threat to the environment where there are toxic released by the hybrid battery. The hybrid cars use implement nickel metal hydride and lithium ion as their battery. Although it is claimed to be less toxic, a substantial amount of carbon monoxide upon usage. The expensive price of the cars itself does not help in leading mankind's life to a better status. Thus, the idea of producing hydrogen from renewable biomass has become all time favourite. Hydrogen is known to have the highest energy content per unit weight among the known gaseous fuel. It contains around 143 GJ per ton of

energy (Boyles, 1984). It also poses no harm since it has the clean combustion potential producing no worse than water vapour and heat energy (Chittibabu *et al.*, 2006). Even though hydrogen can be produced by chemical and physical method, hydrogen production via biological route provides a cost-effective and environmentally harmless option (Sivaramakrishna *et al.*, 2008).

1.3 Objective

The objective of this research is to determine the type of microorganism that is able to utilize oleochemical industry waste and hence produce hydrogen.

1.4 Scopes of Study

This study consists of three main scopes namely:

i. Determining the experimental set up for the anaerobic fermentation system
ii. Testing for the existence of hydrogen gas
iii. Determining growth capability in different medium

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

2.1.1 Hydrogen

Unsustainable fossil fuel triggers the idea of creating and finding the best alternative fuel that will continue to generate the global economy. Fossil fuel being not only non-renewable, it also contribute to air pollution and global warming. It is in fact one of the significant causes that triggers acid rain (Sivaramakrishna *et al.*, 2008). With the intense demand for fossil fuel accompanied by the limited stock for the source of energy has led to various studies of biologically produced hydrogen. Hydrogen has been considered as an ideal and clean energy source for the future since it only produce water upon combustion (Yokoi *et al.*, 1998). Unlike coal and petroleum, hydrogen does not contribute to greenhouse effect, depletion of ozone and acid rain (Chittibabu *et al.*, 2006).

Hydrogen is proven to be high value industrial commodity with wide range of application. Apart from being source of energy, hydrogen has various other uses including a reactant in hydrogenation processes, an oxygen scavenger, and fuel in rocket engines and as coolant in electrical generators. The technology that produces hydrogen biologically using biomass and wastes actually has given a solution to some other problem. It depicts a win-win situation for both unsustainable fossil fuel problem and waste bioremediation. By using the unused, discarded waste from the industry as substrate in producing hydrogen, has indeed helped to properly manage the waste instead of being dumped unattended to. It is from the anaerobic digestion method used to reduce the organic content of waste results into discovery of biogas that consist of ethane and carbon dioxide (Xie *et al.*, 2008). The discovery of these biogas lead to intense research of producing hydrogen via fermentation of industrial waste. The intense need and use for fuel, has triggered the speed of research on alternative fuel. There have been some significant changes in the fuel usage by mankind. The trend of fuel usage has somehow evolved over the years. Meher and Das (2008) have illustrated the change in one simple diagram of how fuel usage had evolved over the years. The diagram can be seen in Figure 2.1.

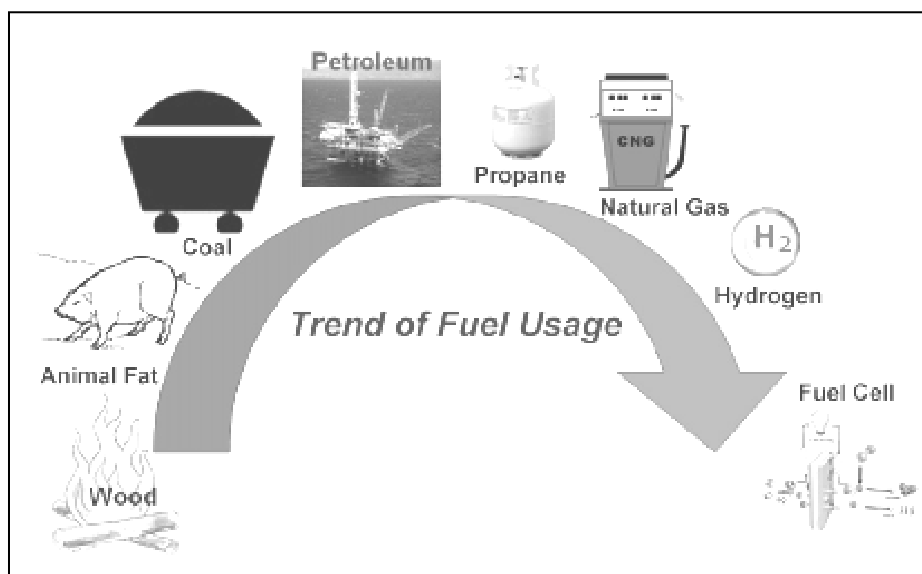


Figure 2.1: Chronology of Fuel Usage by Mankind (Meher and Das, 2008)

2.1.2 Pathways in Producing Hydrogen

There are extensive methods of producing hydrogen whether it is through electrochemical processes, thermochemical processes, photochemical processes, photocatalytic processes or via photoelectrochemical processes. These processes however, do not accomplish the dual goals which are to reduce waste and produce hydrogen at the same time (Han and Shin, 2004). It is logical to categorize the pathways of producing hydrogen into two main categories which is via physical method and biological method. Meher and Das (2008) have summarized the various pathways of hydrogen production from biomass into simple chart as indicated in Figure 2.2 below.

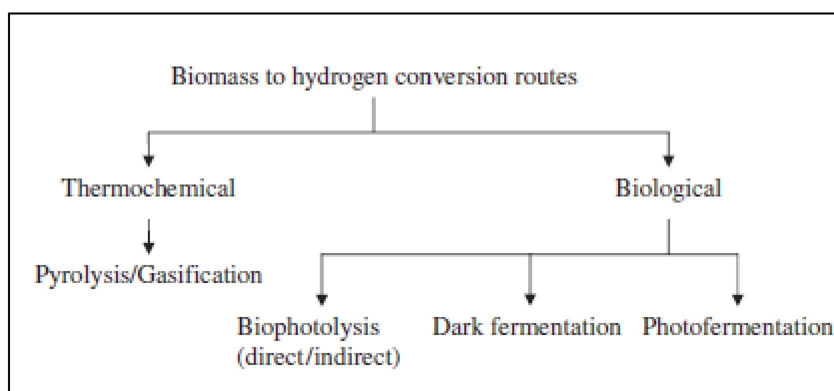


Figure 2.2: Hydrogen Production Routes from Biomass (Meher and Das, 2008)

From the various pathways highlighted above, only two major biological processes that has been focused on over years of research. There are by using photosynthetic organism and one that utilizes fermentative microorganism (Yang *et al.*, 2007). Producing hydrogen utilizing the later microorganism is preferable since it is property for most microorganisms and it is not economically feasible to grow photosynthetic bacteria in such a large volume of photo-bioreactor. Apart from that, fermentative hydrogen production is more advantageous over producing it photosynthetically because it does not rely on the availability of light resources and the transparency of mixed liquor. In synthesizing hydrogen via fermentation,

carbohydrates are favoured substrate over the expensive and hard to get glucose. Glucose substrate yields different amount of hydrogen which then depends on the pathway and its end product (Manish and Banerjee, 2008). The process involves hydrogenase, produces carbon dioxide as well as hydrogen. It is via this method a maximum yield of hydrogen can be obtained with acetate as fermentation product (Hawkes *et al.*, 2002).

2.1.3 Comparison of Physical and Biological Pathway

Recent used methods of producing hydrogen are through chemical and physical methods such as steam reforming and partial oxidation of fossil fuel. These methods are proved to be more expensive and contribute to environmental pollution (Sivaramakrishna *et al.*, 2008).

Via dark fermentation however, hydrogen is produced by anaerobic bacteria that are grown in the dark on carbohydrate rich substrate. In theory, a maximum of 4 moles of hydrogen per mole of glucose will be produced if the fermentation is done in a strict anaerobic condition. The process is as explained by Manish and Banerjee (2008):

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

Apart from hydrogen, there are other main by-products associated with the metabolism of hydrogen fermentation which are acetate, propionate, butyrate and ethanol. These by-products are in fact proven to be valuable and in fact a much priced commodity to the industry.

With all of the pathways mentioned above, many researches up to date have been focusing more on the biological pathway instead of the physical. Among of the biological processes that have caught attention of researches in recent years are photo-fermentation process, two stage fermentation process and bio-catalyzed electrolysis.

In the photo-fermentation process, the substrate which in this case is sugarcane is crushed to produce sugarcane juice. The juice is then fermented in photo-fermentation stage to produce hydrogen. This fermentation produced bagasse as a by-product. The wastewater from the fermentation stage is sent to an anaerobic digester to produce hydrogen (Manish and Banerjee, 2008). The process overview is as shown in Figure 2.3.

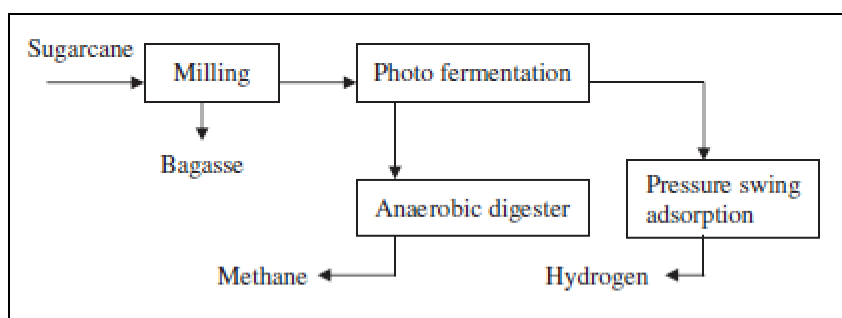


Figure 2.3: Photo-Fermentation Process (Manish and Banerjee, 2008)

Figure 2.4 illustrates the overview process of two-stage fermentation as described by Manish and Banerjee (2008). In this process, the effluent of dark-fermentation is sent to photo-fermentation stage. The hydrogen and carbon dioxide gases produced in both stages are then sent to pressure swing adsorber for separation process.

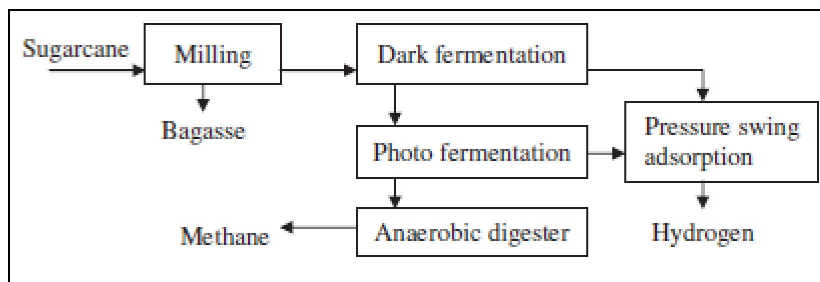


Figure 2.4: Two-Stage Fermentation Process (Manish and Banerjee, 2008)

The schematic diagram in Figure 2.5 shows the process overview in biocatalyzed electrolysis. The effluent from dark-fermentation stage is sent to electrolyzer to produce hydrogen and carbon dioxide. The usage of pressure swing adsorber is avoided since hydrogen and carbon dioxide are produced in different chamber (Manish and Banerjee, 2008).

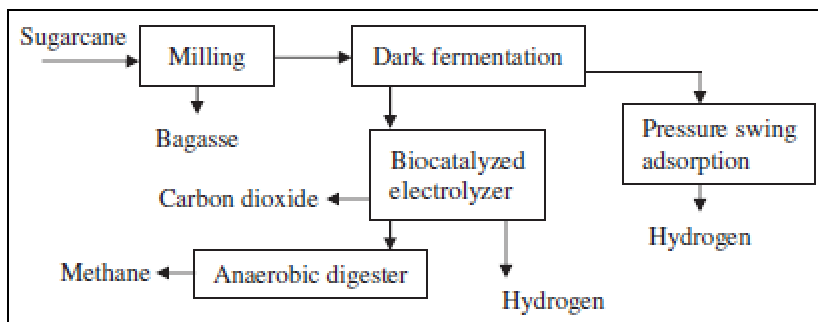


Figure 2.5: Biocatalyzed Electrolysis (Manish and Banerjee, 2008)

Dark fermentation is another process that is well received by some researches. Although it is more economically feasible, the amount of hydrogen yield is not as high in those obtained via photo-fermentation and two stage fermentation (Manish and Banerjee, 2008).

2.2 Oleochemical

2.2.1 Applications of Oleochemical

In this study, oleochemical industry waste is used as substrate for the anaerobic fermentation process. Oleochemical seems to be very promising in producing hydrogen because it is rich in carbohydrates. Glycerol produced form the hydrolysis of oleochemicals are capable to become substrate for hydrogen production. Oleochemicals has been such a great help in production of polymers for quite a sufficient amount of time. There are some researches conducted on oleochemicals' usage to build matrices for natural fiber reinforced plastics (Hill, 2000). Apart than that, oleochemical is proved to be useful and has a high value in industry because of its widespread usage. The applications of oleochemical based product have been summarized in Table 2.1.

Table 2.1: Application of Oleochemical Based Product (Hill, 2000)

Type	**Examples**
Animal feed	Nutritional supplements, emulsifiers for calf milk replacers
Electronics	Wire insulation, insulating varnishes, special purpose plastic components
Food	Emulsifiers and specialties for bread, cakes and pastries, margarine, ice cream and confectionary
Healthcare	Tabletting aid,drugs
Industrial lubricants	General and specialty lubricants, base oils for non-toxic biodegradable lubricants
Leather	Softening, dressing, polishing and treating agents
Metalworking and foundries	Cutting oils and coolants
Wiring	Froth floatation of ores, surface-active
Personal care	Shampoos, soaps, creams and lotions, make up
Paints and coating	Alkyd and other resins
Paper recycling	Removal of printing ink
Plastics	Stabilizers and plasticizers, mould release agents, antistatic and antifogging aid, polymerization emulsifiers
Printing	Printing ink, paper coating, photographic printing
Rubber production	Vulcanizing agents, softeners
Soaps and detergents	Industrial and domestics
Waxes and candles	Ingredients in waxes and polishes

2.3 Substrates

2.3.1 Types of Substrates

Most of the studies that had been carried out over recent years concentrated and focused more on anaerobic fermentation. Substrates used in these studies and experiments are simply sugar and starch which is not economically feasible due to their high cost. Thus more studies are done on anaerobic fermentation by using waste/wastewater from agricultural processes and food industries. These substrates are more preferable since they are in abundant amount and for economic reasons (Yang *et al.*, 2007). A few researches and studies on producing hydrogen via anaerobic fermentation are based on wastes, such as municipal solid waste and wastewaters, sugar manufacturer wastewater, synthetic wastewater, dining hall food waste, alcohol manufacturer wastewater, starch manufacturer waste, rice slurry and cheese processing wastewater (Yang *et al.*, 2007).

Waste utilization has become the favourable substrate in hydrogen generation. Research by Roychowdhury *et al.* (1998) mentioned about the potential of solid wastes and digested sewage sludge to produce large amounts of hydrogen by suppressing the production of methane. To do so, a low volt of electricity is introduced into the sewage sludge. Observation done by Taniho *et al.* (1989) has shown that the removal of carbon dioxide from the culture liquid of *Enterobacter aerogenes* E.82005 will somehow affect the promotion of the yield of hydrogen.

There are various types of wastes that are suitable to become substrate. The substrates are chosen upon their suitability of microorganism introduced into them. Researches done in recent years have used an extensive type of substrate and microorganism via several processes. Among them are used waste water from distillery as an electron donor for photoproduction of hydrogen by *Rhopsodium spaeroides* O.U.001 (Sasikala *et al.*, 1991), usage of mixed culture of photosynthetic and anaerobic bacteria (Miyake *et al.*, 1990), hydrogen production from fermentative bacteria (Ventakaraman *et al.*, 1990) and the usage of starch based residues for

hydrogen production (Roychowdhury *et al.*, 1998). The photosynthetic hydrogen evolution from municipal solid wastes for growth *of R. sphaeroides* RV proposed by Fascetti *et al.* (1995) , has proven that the acidogenesis of fruit and vegetables market wastes gives higher hydrogen evolution rate compared to synthetic medium. In this case it is 100 ml H_2/g dry weight/h by acidogenesis of fruit market and 35 ml H_2/g dry weight/h in synthetic medium. Another substrate favoured by most researches is renewable waste be it from industry or market. Various studies that used mixed microflora as inoculums have succeeded in yielding a substantial amount of hydrogen. Although a vast effort is needed in isolating and identifying the hydrogen producing strain, mixed microflora is proved to be at convenience (Yang *et al.*, 2007 and Sivaramakrishna *et al.*, 2008)

Apart from these proposed substrates there are some other substrates and microorganism used in producing hydrogen. Part of the findings of study done by Das and Veziroglu (2001) has been concluded in Table 2.2.

Table 2.2: Substrates Used and Biologically Producing Microorganism (Das and Veziroglu, 2001)

Organisms used	**Raw materials used**
Photosynthetic bacteria/double photosystem *Oscillatoria sp.* Miami *BG7* *Anabaena cylindrical* *Anabaena variables* *Anabaena* CA	Medium- A except NH_4Cl Nitrogen- starved medium Allen and Arnon medium ASP-2 medium
Photosynthetic bacteria/single photosystem *Rhodopseudomonas capsulate* *Rhodopseudomonas sp* *Rhodopseudomonas sphaeroides* *Rhodopseudomonas palustris* *Rhodobacter sphaeroides* *Rhodospirillium rubrum*	Lactate with other nitrogen source, fermented cow dung Vegetable starch, sugarcane juice, sugarcane wastewater, whey, dairy wastewater Orange processing effluent Straw paper mill effluent, sugar refinery waste Lactic acid fermentation waste, distillery wastewater, lactate from MSW, lactate liquor MSW Organic compounds
Mixed culture *Phormidium valderianum, Halobacterium halobium, E.coli* in 1:1:1 proportion	ASN111 medium devoid of combined nitrogen in TES buffer
Fermentative bacteria (strict anaerobe) *Clostridium butyricum*	Glucose containing medium
Fermentative bacteria (facultative anaerobe)	

Citrobacter intermedium	Cellulose, starch, glucose
Citrobacter freundii	Stillage
Enterobacter aerogenes E82005	Sugar cane
Enterobacter cloacae IIT BT-08	Sucrose containing medium

2.4 Microorganism

2.4.1 Hydrogen Producing Strain Microorganism

Suitable microorganism must be recognized in order to undergo the anaerobic fermentation. Not all types of microorganisms are capable in consuming product and function properly in anaerobic fermentation system. A study done by Das and Veziroglu (2001) has identified and classified the species of microorganisms that are used for hydrogen generation as summarized in Table 2.3.

Table 2.3: Hydrogen Producing Microorganism (Das and Veziroglu, 2001)

Broad classification	**Name of the microorganism**
Green algae	*Scenodesmus obliquus* *Chlamydomonas reinhardii* *Chlamydomonas moewusii*
Cyanobacteria heterocystous	*Anabaena azollae* *Anabaena* CA *Anabaena variabilis* *Anabaena cylindrical* *Nostoc spongiaeforme*

	Nostoc spongiaeforme *Westiellopsis valderianum*
Nonheterocyctous	*Plectonerna boryanum* *Oscillotoria* Miami BG7 *Oscillotoria limnetica* *Synechococcus sp* *Aphanothece halophytico* *Phormidium valderianum*
Photosynthetic bacteria	*Rhodobacter sphaeroides* *Rhodobacter capsulitus* *Rhodobacter sulidophilus* *Rhodopseudomonas sphaeroides* *Rhodopseudomonas palustris* *Rhodopseudomonas capsulata* *Rhodospirillum rubnum* *Chromatium sp.* Miami PSB 1071 *Chlorobium limicola* *Chloroflexu aurantiacus* *Thiocapsa rosespersicina* *Halobacterium halobium*
Fermentative bacteria	*Enterobacter aerogenes* *Enterobacter cloacae* *Clostridium butyricum* *Clostridium pasteurianum* *Desulforibrio vulgaris* *Magashaere esldenii* *Citrobacter intermedius* *Escherichia coli*

Apart from using specific strain of microorganism as proposed by previous researches, mixed microbial communities has become centre of attention in present studies. Utilizing these mixed microbial communities, offer a lot more choices although it requires more time and effort in isolating and identifying the hydrogen producing strain.

2.4.2 Major Enzymes

Enzymes are needed in order to catalyze the fermentation and it helps in producing hydrogen. There are three major enzymes commonly used which are the reversible or classical hydrogenases, the membrane-bound uptake hydrogenases and the nitrogenase enzymes (Das and Veziroglu, 2001).

The reversible or classical hydrogenases oxidize the ferredoxin or other low redox electron carried in a readily reversible reaction. The membrane-bound uptake hydrogenases are capable of taking up hydrogen at low partial pressures but it only produce little or no measurable amount of hydrogen. Nitrogenase enzymes however are capable of evolving hydrogen in the absence of nitrogen. An overview of the mechanism of these enzymes is as shown in Figure 2.6 as depicted by Miyake *et al.* (1990).

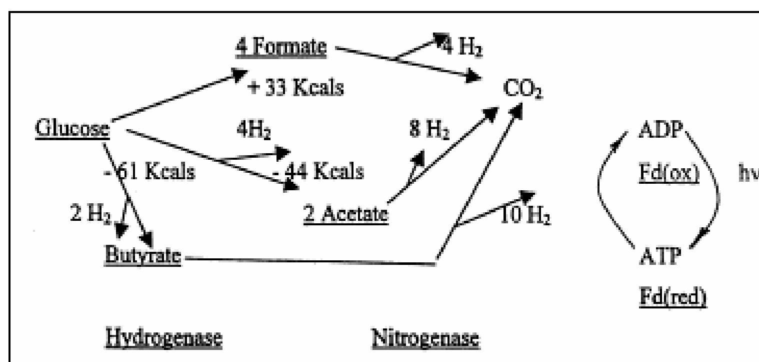


Figure 2.6: Mechanism of Hydrogenase and Nitrogenase Enzymes (Miyake *et al.*, 1990)

2.4.2.1 Reversible or Classical Hydrogenase

Reversible hydrogenase oxidizes ferredoxin or other low redox electron carriers, both natural and artificial, in a readily reversible reaction (Das and Veziroglu, 2001). The hydrogen evolution reaction in green algae is due to such a reversible hydrogenase.

2.4.2.2 Membrane Bound Uptake Hydrogenase

These hydrogenases are able to take up hydrogen at low partial pressures. It reduces a relatively high potential electron acceptor. Even so, it produces little or no measurable hydrogen (Das and Veziroglu, 2001).

2.4.2.3 Nitrogenase Enzymes

Nitrogenase enzymes normally reduce nitrogen to ammonia but it is also capable in evolving hydrogen. This can be done in the absence of nitrogen gas. Among the algae, only blue-green algae such as cyanobacteria possess these enzymes (Das and Veziroglu, 2001). This process is understandable by referring to Figure 2.7.

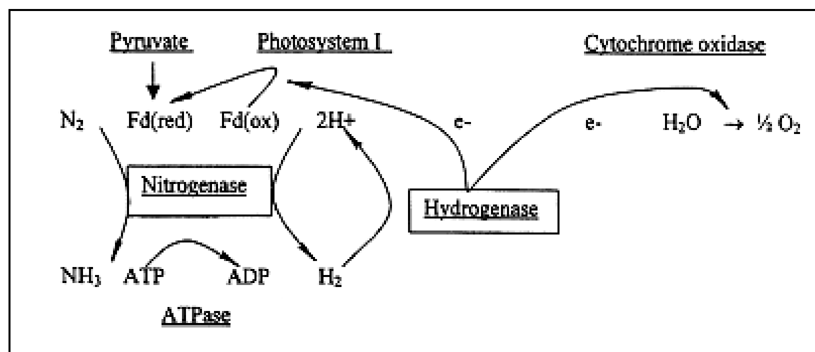


Figure 2.7: Nitrogenase Catalyzed Hydrogen Formation (Das and Veziroglu, 2001)

2.5 Barriers

2.5.1 Barriers in Producing Hydrogen Biologically

Producing hydrogen biologically seems to be more economically feasible and result to a better solution in handling abundant waste. However there are some advantages and disadvantages in making the biological pathway a reality (Das and Veziroglu, 2001). Every type of microorganism used in researches and studies has shown some weaknesses even though it yields a high amount of hydrogen. This is depicted in Table 2.4.

Table 2.4: Advantages and Disadvantages of Different Types of Hydrogen Producing Microorganism (Das and Veziroglu, 2001)

Type of microorganism	**Advantages**	**Disadvantages**
Green algae	➢ able to hydrogen from water ➢ solar conversion energy increased by 10 folds as compared to trees, crops	➢ require light for hydrogen production ➢ Oxygen can be dangerous threat for the system

Cyanobacteria	➢ able to produce hydrogen from water ➢ nitrogenase enzyme mainly produce hydrogen ➢ has the ability to fix nitrogen from atmosphere	➢ uptake hydrogenase enzyme are to be removed to stop degradation if hydrogen ➢ require sunlight ➢ about 30% oxygen present in the gas mixture with hydrogen ➢ oxygen has inhibitory effect on nitrogenase ➢ Carbon dioxide present in the gas
Photosynthetic bacteria	➢ can use different waste (whey, distillery effluents etc) ➢ Can use wide spectrum of light	➢ require light for hydrogen production ➢ fermented broth will cause water pollution problem ➢ carbon dioxide present in the gas
Fermentative bacteria	➢ can produce hydrogen all day long without light ➢ can utilize different crbon sources like starch, cellobiose, sucrose, xylose etc ➢ different types of raw materials can be used ➢ produces valuable metabolites such as butyric acid, lactic acid, acetic acid etc as by-products ➢ anaerobic process means no oxygen limitation problems	➢ fermented broth require treatment before disposal since it may cause water pollution problem ➢ carbon dioxide present in the gas

Not only there are some disadvantages concerning the microorganism used, there are also some other barriers that exist in biologically produce hydrogen. The barriers are as depicted by Das and Veziroglu, 2001 in the Table 2.5.

Table 2.5 Barriers and Solution in Producing Hydrogen Biologically (Das and Veziroglu, 2001)

Type of barrier	**Barrier**	**Possible solution**
Organism	➢ bacteria do not produce more than 4 mol of hydrogen naturally	➢ isolate more novel microbe and combinational screens for hydrogen production rate yields and durability ➢ genetic manipulation of established bacteria
Enzyme (hydrogenase)	➢ hydrogenase overexpression is not stable ➢ oxygen sensitivity ➢ hydrogen feed back inhibition	➢ greater understanding of the enzyme regulation and expression ➢ mutagenic studies ➢ low hydrogen partial fermentation
Feedstock	➢ high cost of suitable feed stock(glucose) ➢ low yield using renewable biomass	➢ renewable biomass as feedstock ➢ co-digestion use of microbial consortia which can increase the yield
Strain	➢ lack of industrial suitable strain	➢ development of industrially viable strain/consortia

Process	➢ commercially feasible product yield ➢ incomplete substrate utilization ➢ sustainable process ➢ sterilization	➢ hybrid system ➢ link fermentation to a second process that makes both economically possible ➢ application and utilization of fermentation tools such as continuous culture ➢ development of low cost stream sterilization technology/process that can bypass sterilization
Reactor	➢ lack of kinetics/appropriate reactor design for H_2 production ➢ light intensity in case of photo bioreactor	➢ incorporation of process engineering concepts to develop a suitable reactor for the defined process ➢ flat panel or hollow tube reactor can be employed
Thermodynamic	➢ thermodynamic barrier	➢ reverse electron transport to drive H_2 production to past barrier
Hydrogen	➢ H_2 purification ➢ Storage	➢ selection absorption of CO_2/H_2S ➢ basic studies on H_2 storage

Although there are challenges underlying ahead of producing hydrogen biologically, it still holds a future for generation ahead. The barriers had been identified and solutions are being proposed to overcome them. Thus, it is possible to create a future fuel that generates millennium economy out of hydrogen.

CHAPTER 3

METHODOLOGY

3.1 Introduction

The best available technique in producing hydrogen from oleochemical industry waste is through anaerobic fermentation. This method is highly likely to be feasible since the best producers of hydrogen gas via fermentation known are anaerobic. Since the fermentation is going to be done in a strict anaerobic manner, the experimental set up must be able to portray the anaerobic fermentation system. Not only the system is able to function in anaerobic condition, it will have to able to prove the presence of hydrogen gas. The concept of the hydrogen detection system is based on the theory proposed by Manish and Banerjee (2008) in which carbon dioxide and hydrogen are formed from anaerobic fermentation. The idea of this study is to determine the hydrogen producing microorganism that is able to utilize the oleochemical industry waste. Thus, mixed culture would be inoculated into the fermentation broth. Upon the fermentation, if there is any existence of hydrogen, the hydrogen producing microorganism would be isolated and identified.

3.2 Materials and Method

3.2.1 Microorganism and Culture Conditions

The cultures used in this experiment were mixed microflora obtained from both treated and untreated waste of an oleochemical company known as Felda Vegetable Oil Sdn Bhd. The wastes were stored in 2 L of Schott bottles and stored inside the fridge at 4°C. The cultures were activated by transferring 10 mL of the waste into 100 mL of medium. The medium and inoculums differ for each trial. The combinations of medium and inoculums are stated in Table 3.1. The cultures were activated anaerobically in the medium inside a 250 mL Schott bottle for 24 hours at 37°C. Then, 50 mL of this culture were used as inoculums. In adjustment stage trials, K-solubilizer enhancer microorganisms obtained from biofertilizers project studies were used. The activation of the microorganism is exactly in the same manner of the mixed microflora.

Table 3.1: Combination of Inoculums and Medium

Trials	Medium	Inoculums
1	TYA medium	K-solubilizer enhancer microorganism
2	TYA medium	K-solubilizer enhancer microorganism
3	TYA medium	K-solubilizer enhancer microorganism
4	TYA medium	Untreated oleochemical waste
5	TYA medium	Untreated oleochemical waste
6	Untreated oleochemical waste (unfiltered)	Untreated oleochemical waste
7	Untreated oleochemical waste (filtered)	Untreated oleochemical waste

8	TYA medium	Treated oleochemical waste
9	Treated oleochemical waste	Untreated oleochemical waste
10	TYA medium	Untreated oleochemical waste
11	Untreated oleochemical waste	Untreated oleochemical waste
12	Untreated oleochemical waste	Treated oleochemical waste
13	TYA medium	Untreated oleochemical waste
14	TYA medium	Untreated oleochemical waste

3.2.2 Medium Preparation

There were three types of medium used in this study. They are TYA medium, autoclaved untreated oleochemical waste and autoclaved treated oleochemical waste. The TYA liquid medium used contains 4 g glycerol, 2 g yeast extract, 10 mg iron (II) sulphate heptahydrate, 0.5 g kalium di-hydrogen phosphate and 0.3 g magnesium sulphate heptahydrate per liter distilled water. The pH of the medium was adjusted to 6.5 with 1 M natrium hydroxide and sterilized at 121°C for 15 minutes. The agar medium is also needed in the experiment to grow the possible hydrogen producing microorganism for identification. The agar medium was prepared by dissolving 182 g of nutrient broth in 1 L of distilled water. The agar was then sterilized at 121°C for 15 minutes before being transferred onto agar plates. The untreated and treated waste obtained from the oleochemical company was stored inside the refrigerator at 4°C overnight to allow sedimentation of large particles. Then it is filtered using nylon membrane of 0.45 µm. After that, it is autoclaved at 121°C for 20 minutes. Only then it is used as medium according to the combination stated in Table 3.1.

3.2.3 Hydrogen Fermentation

The hydrogen fermentation was done in batch. The gas production experiments were done inside five liters round bottom flask with inlet and outlet tubing for sparging nitrogen and collecting gas. The fermentation system was carried out in trials as stated in Table 3.1. The fermenter flask was filled with 500 mL of medium before being sparged for 5-10 minutes prior inoculation. Then 10% of activated culture was inoculated into the fermenter flask. The culture was then left for 48 hours for fermentation at either 28°C or 37°C. The fermenter flask was connected to either hydrogen detection system 1, 2 or 3 for different temperature or detection system.

3.2.4 Hydrogen Detection System

Theoretically, anaerobic fermentation produces hydrogen and carbon dioxide gas. In this study, the hydrogen gas was detected using a classical analytical method which is acid displacement system as shown in Figure 3.1. The detection system 1 shown in Figure 3.1 is for trials carried out at temperature of 28°C. The flask filled with broth culture was wrapped around with aluminum foil to avoid growth of algae due to photosynthetic reaction with light. It was placed onto magnetic plate stirrer with the stirrer turned on to ensure constant mixing of the broth. The gas mixtures of hydrogen and carbon dioxide produced from the fermentation were passed through sodium hydroxide solution of 5M. Carbon dioxide gas reacted with sodium hydroxide to form sodium carbonate. Then the final free carbon dioxide and hydrogen was detected by how much the hydrochloric acid displaced. A low pH solution of 0.0326M was used instead of distilled water to avoid the absorption of hydrogen gas during hydrogen gas production.

Figure 3.1: Hydrogen Detection System 1

Hydrogen detection system 2 applied the same detection concept as in hydrogen detection system 1. The gas mixtures were passed through 5M of sodium hydroxide and hydrochloric acid of 0.0326M. The difference between system 1 and 2 is the temperature. In hydrogen detection system 2, the flask was placed inside a water bath. The water bath was set at 37°C and mixed well constantly at the speed of 40 RPM. Figure 3.2 shows the set up for hydrogen detection system 2. Apart from that, pH indicator is added to the 5M sodium hydroxide solution. The pH indicator showed some colour changes in which it turned to indigo (pH 12) when carbon dioxide is being absorbed into the solution. The colour changes in pH indicator were depicted in Figure 3.3.

Figure 3.2: Hydrogen Detection System 2

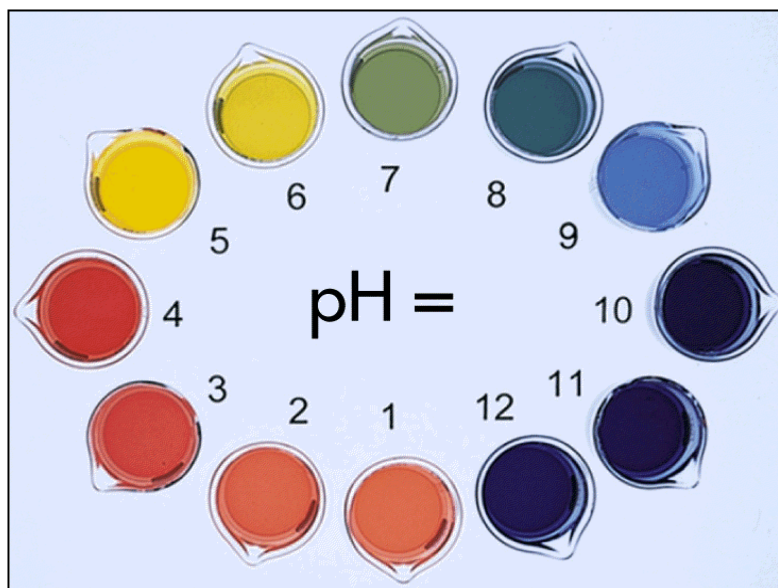


Figure 3.3: Colour Changes in pH Indicator

The third sytem used the hydrogen 'pop' test concept. In this system the fermenter flask was connected to a sampling bag via rubber tubing as shown in Figure 3.4. As the fermentation in progress, all of the gas released was collected inside the sampling bag. After 48 hour fermentation, the sampling bag was held with long stick over burning candle. Presence of hydrogen inside the bag would somehow react with the flame from the candle and burst the sampling bag. Bursting of the sampling bag proved the presence of hydrogen.

Figure 3.4: Hydrogen Detection System 3

3.3. Optical Density of Broth Culture

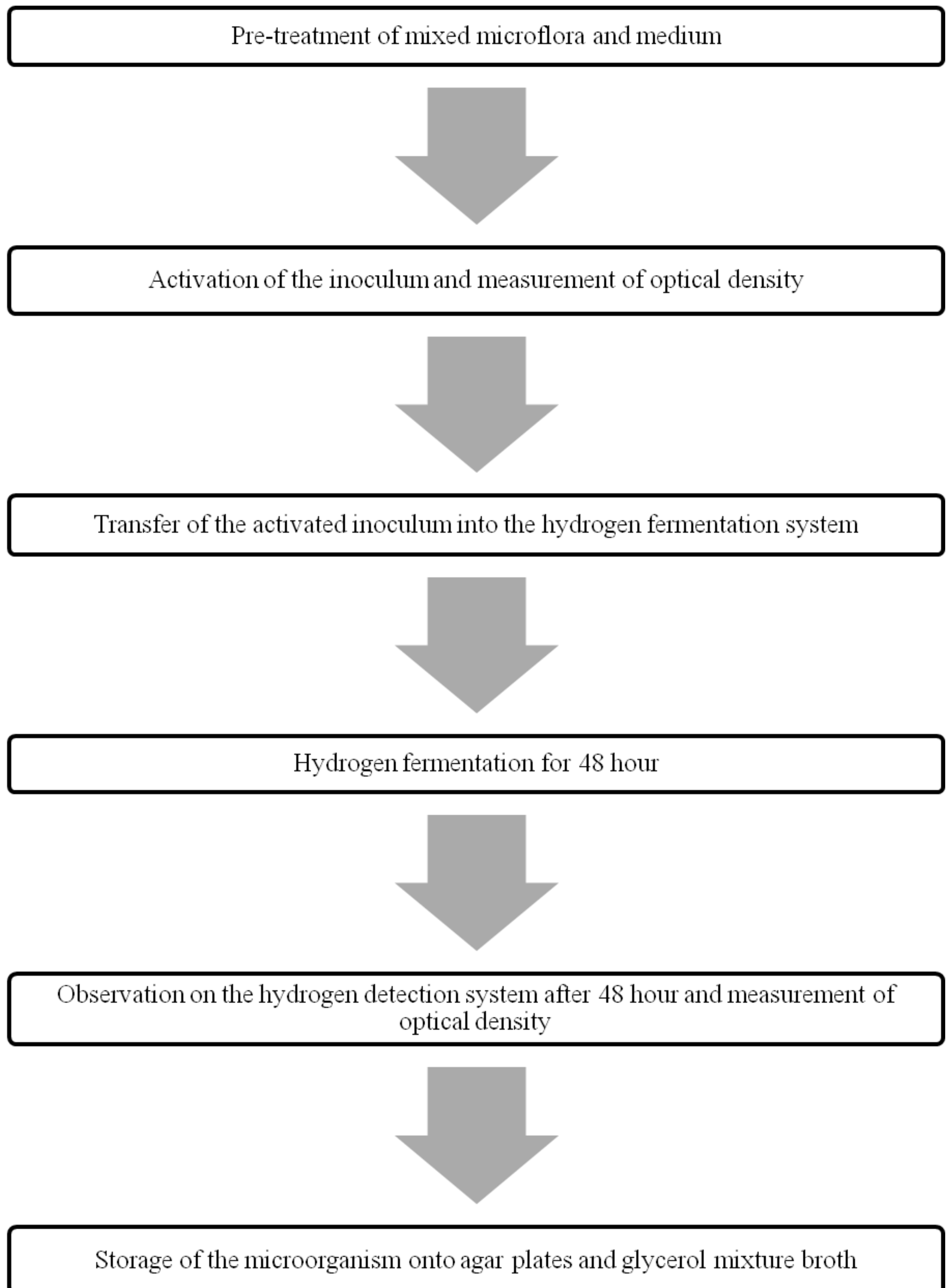
The main idea to read the optical density of the broth culture was to detect whether the microorganism utilize the medium. Differences in the reading prior fermentation and after fermentation indicate that the microorganisms are utilizing the medium as planned. Upon inoculation of the culture in the activation stage, 5 mL of the broth culture was filled into a cuvette. One cuvette was filled with distilled water is used as blank. Then both blank and the broth culture were measured for its absorbance using ultra-violet spectrometer at wavelength of 660nm. Same procedures were carried out once the 48 hour hydrogen fermentation completed.

3.4 Storage of Microorganism

This process was carried out once there are differences in the optical density reading. Once there are differences in the readings, then the broth culture need to be transferred onto agar plates for identification process and storage. The identification process is to identify the hydrogen producing microorganism. The broth culture was transferred onto the agar plates using aseptic technique of streaking agar. Before being transferred onto agar plates, the broth culture needed to be diluted at first. Six beakers were filled with 40 mL of the TYA medium. Then 10% of the broth culture was transferred into the first beaker. After mixing it well, 10% of the culture was transferred into the second beaker. Same procedures were carried out for the whole six beakers. For each of the dilution done, a replicate of agar plates were being done. Once the culture was transferred onto the agar plates, it was then incubated at 30^oC for 48 hour. Upon finishing the incubation process, the plates were then stored inside the refrigerator at 4^oC.

Apart from that, the microorganism was also stored in broth form. This was done by mixing 80 mL of the fermentation broth with 20 mL of pure autoclaved glycerol. The percentage of glycerol used was 20% v/v. The mixture was mixed well before being stored inside the refrigerator at -80^oC.

3.5 Summary of Methodology



CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

This chapter depicts the results and observation obtained from the entire study that has been carried out. Most of the results obtained from this study were based on observations and measurement of the optical density. Throughout the span of this study, a total of 14 trials have been carried out. Four of the initial trials were categorized as adjustment stage. It was within these four trials, the hydrogen detection system has been set up and adjusted several time in order to come up with the desired system. All of the hydrogen detection systems used in this study has been perfected and tested during the adjustment stage.

4.2 Result and Discussion

4.2.1 Result on Hydrogen Detection System

All of the observed results were tabulated in Table 4.1 as shown. The medium and inoculums combination of each trials were mentioned. The table also states the hydrogen detection system used to test the presence of hydrogen gas.

Table 4.1: Observation on Biohydrogen Detection System

Date	Trial	Condition	Observation
27/11-28/11	1	Medium: TYA medium Inoculum: K-solubilizer enhancer microorganism System: 1 (using hydrochloric acid of 2M)	-adjustment stage -no acid displacement -acid turned yellow in colour
3/12-4/12	2	Medium: TYA medium Inoculum: K-solubilizer enhancer microorganism System: 1	-adjustment stage -no water displacement
5/12-9/12	3	Medium: TYA medium Inoculum: K-solubilezer enhancer microorganism System: 1	-water displacement occurred at average rate
11/12-12/12	4	Medium: TYA medium Inoculum: untreated oleochemical waste System: 1	-rapid water displacement
31/12-02/01	5	Medium: TYA medium Inoculum: untreated oleochemical waste System: 1 (adjusted)	-no acid displacement
07/01-09/01	6	Medium: autoclaved untreated oleochemical waste Inoculum: untreated oleochemical waste System: 1	-no acid displacement occurred
14/01-16/01	7	Medium: autoclaved untreated oleochemical waste Inoculum: untreated oleochemical waste	-no acid displacement occurred -pH indicator inside the base changed

		System: 2	colour to dark indigo
21/01-23/01	8	Medium: TYA medium Inoculum: treated oleochemical waste System: 2	-no acid displacement occurred -pH indicator inside the base changed colour to dark indigo
29/01-31/01	9	Medium: autoclaved treated oleochemical waste Inoculum: untreated oleochemical waste System: 2	-no acid displacement occurred -no colour changes on pH indicator in the base
11/02-13/02	10	Medium: autoclaved untreated oleochemical waste Inoculum: untreated oleochemical waste System: 2	-no acid displacement occurred -pH indicator inside the base changed colour to dark indigo
18/02-20/02	11	Medium: TYA medium Inoculum: untreated oleochemical waste (aerobic) System: 3	-sampling bag was full with gas -two bags were used
25/02-27/02	12	Medium: TYA medium Inoculum: untreated oleochemical waste(anaerobic) System: 3	-no gas was collected inside the sampling bag
04/03-06/03	13	Medium: TYA medium Inoculum: untreated oleochemical waste(aerobic) System: 3	-no gas was collected inside the sampling bag
04/03-06/03	14	Medium: TYA medium Inoculum: untreated oleochemical waste(anaerobic) System: 3	-no gas was collected inside the sampling bag

4.2.1.1 Discussion on Hydrogen Detection System

The first four trials that were carried out in this study were categorized as the adjustment stage. Details of the changes and adjustment done were to be discussed. The first trial that was carried out in this study used a highly concentrated acid in its hydrogen detection system 1. However there were no displacements of acid occurred but the colour of the acid changed to yellow. This might due to some reaction occurred between the gas released and the acid itself. Since there were no acid displaced, it was caused by the length of the glass tubing that went inside the measuring cylinder itself. The mouth of the glass tubing was too near to the mouth of measuring cylinder. With such great pressure difference between the outer and inner of the measuring cylinder, the gas was not able to displace the acid out of measuring cylinder itself.

Since the hydrogen detection system 1 required a lot of acid, the usage of acid was then replaced with distilled water. This was due to the fact, distilled water is safer and easier to handle compared to a highly concentrated acid. Since the second trial showed no displacement, it was then confirmed that the length of the glass tube that went inside the measuring cylinder was the cause. The problem was solved by elongating the tubing. A piece of rubber tubing was attached to the glass tubing so that the mouth of the tubing would be far enough from the mouth of measuring cylinder. This overcame the great pressure different problem.

With the tubing adjusted, Trial 3 and Trial 4 that were carried out showed some promising result. There were some water displacement occurred although in Trial 4 had shown some rapid water displacement. The rapid water displacement occurred in Trial 4 was highly due to pressure build up during sparging the flask with nitrogen. Although there was some water displacement occurred in these two trials, presence of hydrogen was not affirmative. This was due to the fact that hydrogen dissolved in distilled water. Thus the displacement of water in both trials might due to the pressure build up occurred during sparging of the flask.

Since previous studies and researches concluded that hydrogen dissolve in distilled water, the system is adjusted according to the classical analytical method of acid displacement system. Any displacement of acid would confirm the existence and presence of hydrogen since carbon dioxide and other gases were absorbed by sodium hydroxide. However, both Trial 5 and Trial 6 did not showed any displacement on the acid and changes on the base.

Throughout Trial 7 to Trial 10, the hydrogen fermentation was carried out at $37^{o}C$. This was done since most of the researchers before claimed that most hydrogen producing microorganism' optimum temperature is at $37^{o}C$. Few drops of pH indicator were also being added into the sodium hydroxide. This is to determine whether there was any carbon dioxide absorbed into the base. There were some colour changes in Trials 7, 8 and 10 respectively but none in Trial 9. This is a clear indication that the medium-inoculum combination used in Trial 9 is not suitable for this study since there were no gas released during fermentation. The colour of pH indicator turned into dark indigo of pH 12 from navy blue of pH 10 which indicates that the base has absorbed some carbon dioxide.

Hydrogen 'pop' test concept was applied on Trial 11 to Trial 14. There were gases produced from fermentation in Trial 11. The gases were collected in gas chromatography sampling bag as shown in Figure 4.1. The gas that was collected inside the GC sampling bag in Trial 11 was held close to a match. When the test was carried out, a faint 'pop' sound was heard.

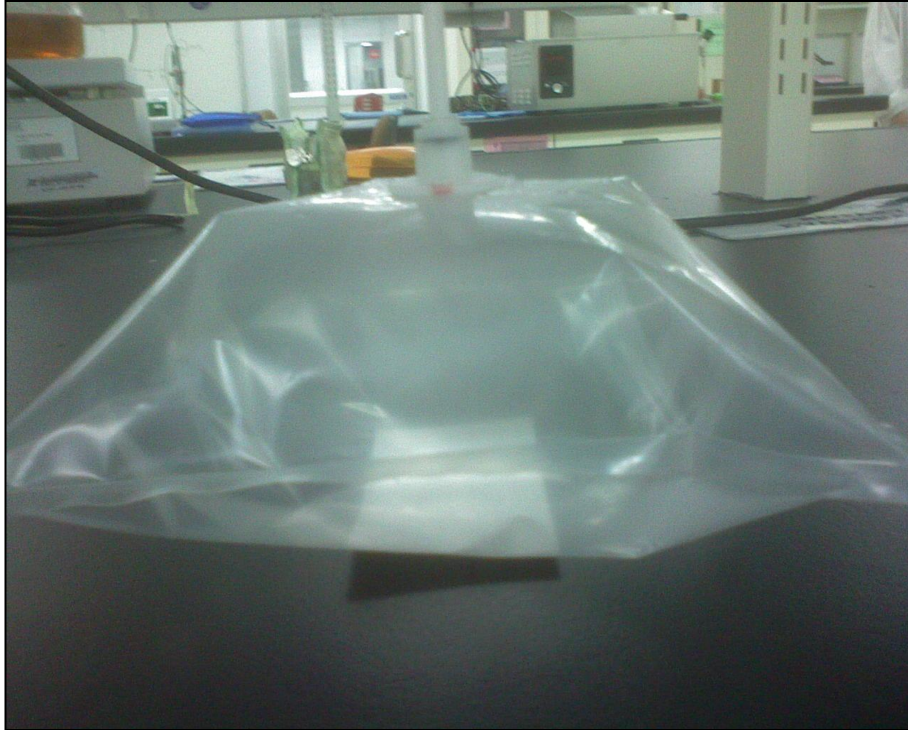


Figure 4.1: Gas Collected in Trial 11

In order to confirm the sound heard in Trial 11, repetition was made. But due to some technical problems on the water bath and the power was shut down for almost half a day, there were no gases collected in the following trials. This caused the result obtained in Trial 11 unconfirmed and arguable.

4.2.2 Result and Discussion on Optical Density Measurement

Readings on optical density at the initial and final phase of each trial is crucial since it helps in determining whether the microorganisms were able to utilize the medium given. With three different types of medium used, it was required to recognize which medium that the microorganism were able to grow in hence have the capability to induce the microorganism to produce hydrogen. All of the readings on optical density of trials starting from Trial 5 are tabulated in Table 4.2.

Table 4.2: Optical Density Measurement at 660 nm. The final OD value was taken after 48 hours of fermentation

Trial	Initial Optical Density (OD)	Final Optical Density (OD)
5	0.119	0.399
6	0.208	0.417
7	0.136	0.407
8	0.015	0.805
9	0.149	0.117
10	0.062	1.297
11	0.208	1.389
12	0.132	1.209
13	0.019	1.582
14	0.016	1.560

As seen in Table 4.2, a huge difference between the initial and final readings of the optical density throughout Trial 10 to Trial 14 showed that the microorganisms introduced into the medium were able to utilize them well. The situation however differed in Trial 5 to Trial 9 where the readings differences were not that huge. This might due to the unsuitable temperature used in these trials which was at $28^{o}C$. The reading in Trial 9 even showed a lower reading than the initial indicating that there were no microorganisms survived in the medium used. Thus it was a clear indication that the medium used in Trial 9 was not suitable for any microorganism growth.

4.2.3 Result and Discussion on Broth Colour

Starting from Trial 7 onwards, pictures of the broth before and after undergoing fermentation were taken. This was to support the optical density measurement taken in the trials. The changes in colour of the broth culture showed the capabilities of the microorganism to utilize and grow upon the medium used. A

muddy and cloudy broth culture showed that the microorganisms were able to grow in the medium introduced to them.

Figure 4.2 showed the initial and final pictures of broth culture in Trial 7. The autoclaved untreated oleochemical waste used as medium in this trial was filtered beforehand. Unlike in other trials, the final broth culture in Trial 7 turned into a substance very similar with oil regarding its colour and viscosity.

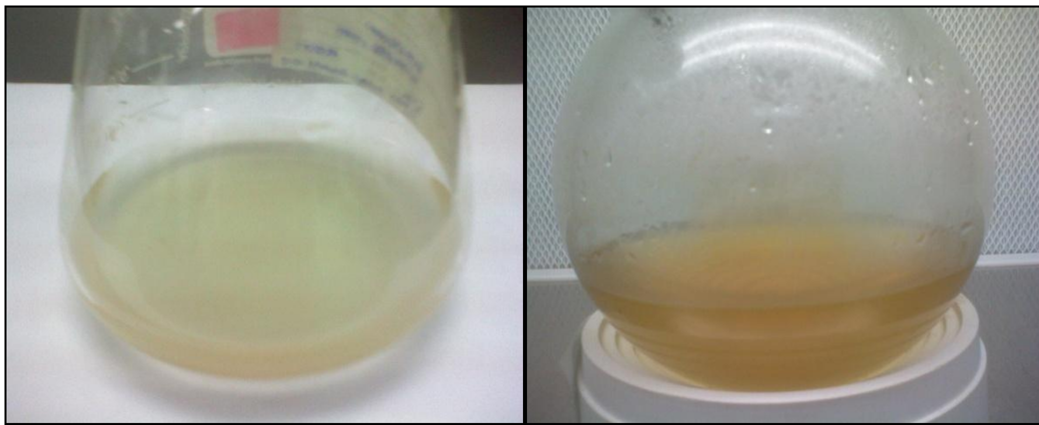


Figure 4.2: Trial 7 Broth Culture. On the left: Broth culture before fermentation. On the right: Broth culture after 48 hours of fermentation.

The colour of the broth culture in Trial 8 did not change greatly than the initial broth culture. Both initial and final broth culture still retained its colour. It was only in the final broth culture, the colour is a little cloudy than the initial. This is as shown in Figure 4.3.

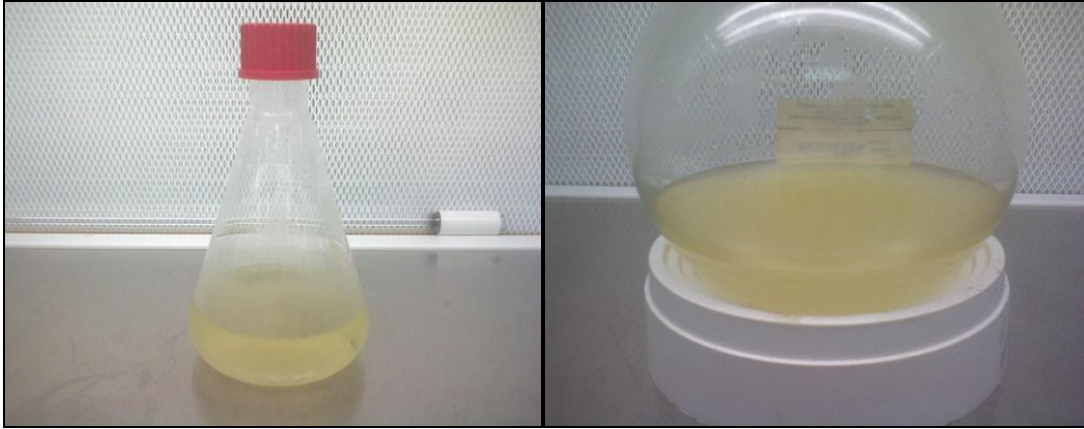


Figure 4.3: Trial 8 Broth Culture. On the left: Broth culture before fermentation. On the right: Broth culture after 48 hours of fermentation.

In Trial 9, the colour of the final broth culture was clearer than the initial broth culture. This is as shown in Figure 4.4. This explained the decreased optical density reading in Trial 9 indicating that there were no microorganisms grew in the medium used.

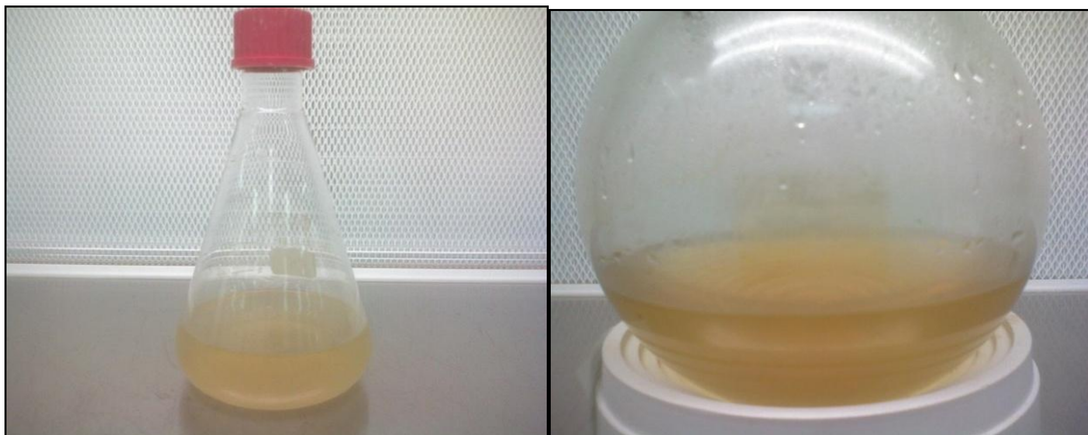


Figure 4.4: Trial 9 Broth Culture. On the left: Broth culture before fermentation. On the right: Broth culture after 48 hours of fermentation.

The broth in trials 10 to 14 resulted to the same colour. In all five final trials, the final broth turned into a cloudy colour. It was a clear indication that the mixed microflora introduced into the trials was able to utilize the mediums used in trials 10 to 14. Thus it explained the high measurement of the final optical density in all five final trials. The colour changes of the trials are shown in Figure 4.5.

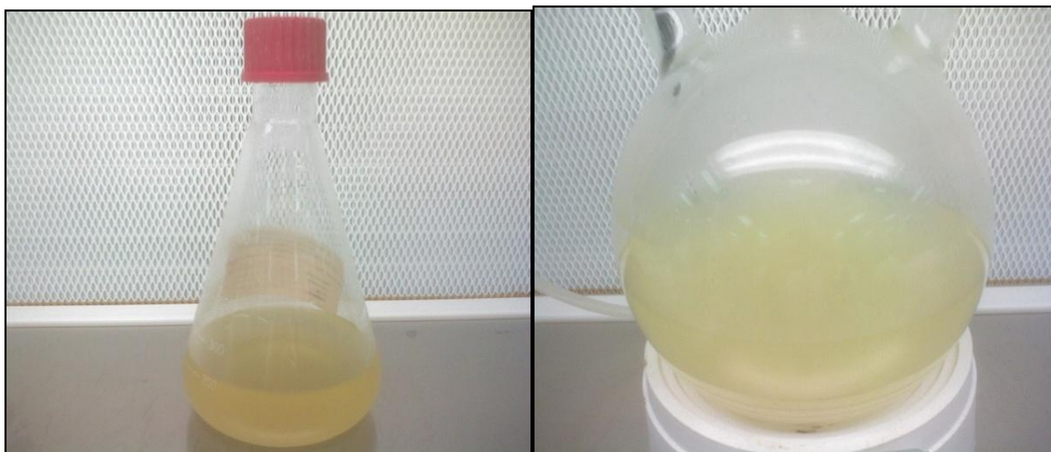


Figure 4.5: Trial 10-14 Broth Culture. On the left: Broth culture before fermentation. On the right: Broth culture after 48 hours of fermentation.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Based on the result obtained, the hydrogen producing microorganism has not as yet been identified. According to study done by Das and Veziroglu, 2001, the most hydrogen producer among anaerobic microorganism is from *Clostridium sp.* So it is most likely the microorganism that is able to utilize the oleochemical waste is from *Clostridium sp.* From all of the three detection systems set up, the hydrogen detection system 2 and 3 showed some promising results. The results obtained from the trials conducted were not conclusive and concrete due to the little amount of gas released from the fermentation. However, the hydrogen detection system did showed some promising result. With a substantial amount of gas produced from fermentation, the system would have given a much concrete and conclusive result. This can be seen in Trial 11, in which two sampling bags were required to collect the gas produced from the fermentation done. Apart from that, TYA medium and autoclaved untreated oleochemical waste proved to be suitable as substrate for anaerobic fermentation. At the same time, these two mediums were able to culture mixed microflora contained hydrogen producing microorganism.

5.2 Recommendations

5.2.1 Usage of Gas Chromatography

In order to obtain a conclusive and affirmative result in this study, usage of gas chromatography is highly recommended. As when this study is conducted, the gas collected in the trials were not able to be tested using gas chromatography. This is due to unavailability of hydrogen detection column required. It is suggested to supply the gas chromatography with the suitable column beforehand since the purchasing of the column would take some time. The column suitable for the hydrogen detection is 6 feet stainless steel column packed with porapak Q (80/100 mesh). The operational temperatures of the injection port, the oven and the detector are 100°C, 50°C and 100°C respectively. With the usage of gas chromatography, the results obtained would not be arguable.

5.2.2 Usage of Anaerobic Jar/Chamber

The need of anaerobic jar or chamber is considered crucial in this study. This is because the fermentation carried out in this study is anaerobic fermentation. From the researches carried out before, many researchers had concluded that the best hydrogen producing microorganisms are strict anaerobic microorganisms. In order to allow the hydrogen producing microorganism to function at its optimum condition, a strict anaerobic environment must be created. Thus, the use of anaerobic jar is crucial after sparging the fermenter flask with nitrogen to ensure its anaerobic condition.

5.2.3 Adjustment on the Fermenter Flask

The fermenter flask used in this study is a 2 L round bottom flask with three necks. During the fermentation, all of the inlets need to be closed shut to avoid any loses of gases produced from the fermentation. If any of the inlets are not closed properly with air tight stopper, the possibility of gases loses is very likely. Loses of gases is very crucial since it will greatly affect the result of the trials that has been carried out. At the same time, it is also to ensure that the fermentation is done in a strict anaerobic fermentation. Once the fermenter flask was sparged with nitrogen, the flask needed to be connected to the hydrogen detection system quickly in order to minimize the exposure to oxygen.

Further adjustment on the fermenter flask is highly recommended in order to prevent gases produced from the fermentation to escape. Adjustments recommended for the fermenter flask is as shown in Figure 5.1.

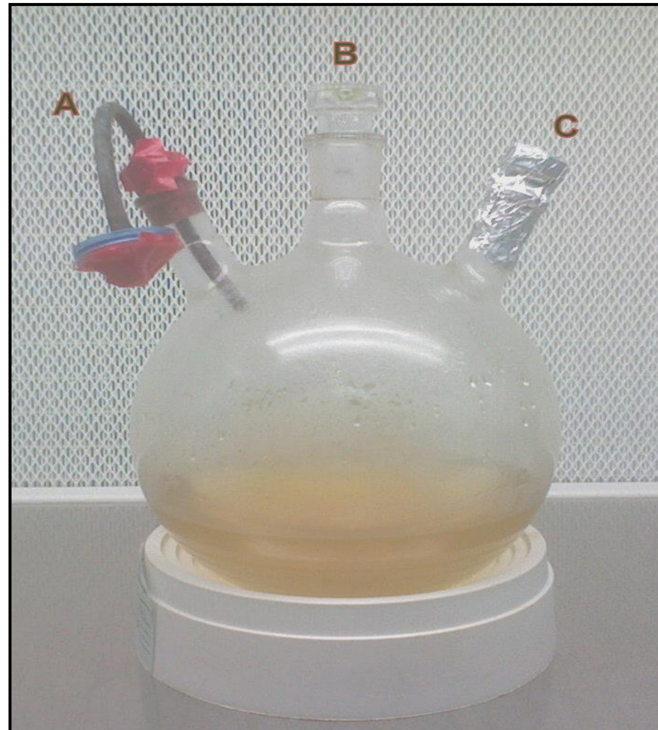


Figure 5.1: Fermenter Flask

The adjustments required are stated below:

- A : Ensure the rubber tubing that goes through the rubber stopper is air tight. Another short length of rubber tubing is connected to the filter to allow clamping. The clamp will not allow any gas transfer in and out of the flask.
- B : Replace the glass stopper with a connecter equipped with valve that will make connection of the flask and the hydrogen detection system easier without having to expose the flask to oxygen. The connector suggested is a 24/26 glass connector as shown in Figure 5.2.

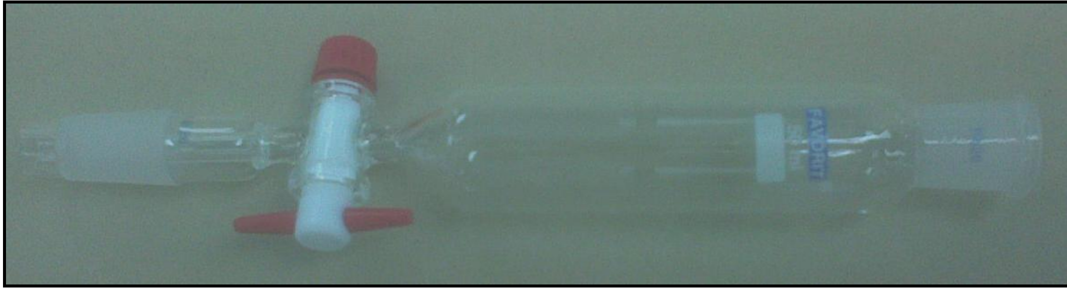


Figure 5.2: Glass Connector

5.2.4 Minimize the Length of Tubing

With the small amount of gas produced from the fermentation taking account of loses of gases, the length of tubing used in the hydrogen detection system might be too long. The length of tubing for the gases to travel to base and acid solution might be too much. Thus, the gases became droplets along the tubing instead of reacting with the base and displace the acid in hydrogen detection system 2. The length of tubing that connects the base and acid solution need to be shortened in order to allow the gases produced from the fermentation to react and displace the acid.

5.2.5 Repetition on Trials

Repetitions on certain trials are highly suggested. This is to ensure the validity of results observe and obtain from each trials. Although repetitions have been carried out in this study, it is still not enough. This is because the repetitions were done during adjustments on the system. Thus, to ensure and confirm the results obtained, it is advisable to repeat the trials under the same circumstances without being obstruct with any adjustments on the system.

5.2.6 Usage of Various Single Cultures

It is recommended and a lot more flexible when several of single cultures are being used. Although it is time consuming, but the usage of several single cultures will help by producing more desired yield. Application of known hydrogen producing microorganism in this study will allow more focus and attention in developing a reliable hydrogen detection/collection system. Apart from that, the gas produced will be most likely to be hydrogen. At the same time, isolation and identification of microorganism used would not be necessary.

REFERENCES

Boyles D. Bioenergy technology-thermodynamics and costs. New York: Wiley; 1984.

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

Das D and Veziroglu T. N. (2001). Hydrogen production by biological processes: a survey of literature. *International Journal of Hydrogen Energy*. 26:13-28.

Fascetti E. and Todini O. (1995). *Rhodobacter sphaeroides* RV cultivation and hydrogen production in one and two-stage chemostat. *Application Microbioal Biotechnology*. 44:300–305.

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

Hawkes F.R., Dinsdale R. , Hawkes D.L. and Hussy I. (2002). Sustainable fermentative hydrogen production: challenges for process optimization. *International Journal of Hydrogen Energy*. 27:1339-1347.

Hill K. (2000). Fats and oils as oleochemical raw material. *Pure Appl. Chem*. 72: 1255-1264.

Kalia V.C, Jain S.R, Kumar A. and Joshi A.P. (1994). Fermentation of biowaste to H_2 by *Bacillus licheniformis*. *World Journal of Microbiology and Biotechnology*. 10: 224-227.

Kumar N and Das D. (2000). Production and purification of alpha-amylase from hydrogen producing Enterobacter cloacae IIT-BT 08. *Bioprocess Engeneering*. 23:205–208.

Manish S. and Banerjee R. (2008). Comparison of biohydrogen processes. *International Journal of Hydrogen Energy*. 33:279-286.

Meher K. S. and Das D. (2008). Biohydrogen as a renewable energy resource-Prospects and potentials. *International Journal of Hydrogen Energy*. 33:258-263.

Miyake J. Application of photosynthetic systems for energy conversion (1990). *Hydrogen energy progress VIII. Proceedings 8th WHEC, Hawaii. New York: Pergamon Press.*755–764.

Penfold D.W., Forster C.F and Macaskie L.E. (2003). Increased hydrogen production by *Escherichia coli* strain HD701 in comparison with the wild type parent strain MC4100. *Enzyme and Microbial Technology*. 3:185-189.

Roychowdhury S., Cox D., Nag M., and Cipico P.E. (1998). Continuous generation of hydrogen from sewage sludge. *Hydrogen energy progress XII. Proceedings of 12th WHEC, Argentina.* 533–41.

Sasikala K., Ramana V.C. and Subrahmanyam M. (1991). Photoproduction of hydrogen from wastewater of a lactic acid fermentation plant by a purple non-sulfur photosynthetic bacterium *Rhodobacter sphaeroides* O.U. 001. *Indian J Exp Biology*. 29:74–75.

Sivaramakrishna D., Sreekanth D., Himabindu V. and Anjaneyulu Y (2008). Biological hydrogen production from probiotic wastewater as substrate by selectively enriched anaerobic mixed microflora. *Renewable Energy*. 45:1-4.

Sung S., Bazylinski D. A and Raskin L. (2003). Biohydrogen production from renewable organic waste. Hydrogen, Fuel Cells and Infrastructure Technologies. FY 2003 Progress Report.

Tanisho S., Tu H.P. and Wakao N. (1989). Fermentative hydrogen evolution from various substrates by *Enterobacter aerogenes*. *Kakkokogaku*. 67:29-34.

Venkataraman C, and Vatsala T.M. (1990). Hydrogen production from whey by phototrophic bacteria. *Hydrogen energy progress VIII. Proceedings of 8th WHEC, Hawaii. New York: Pergamon Press*.781–788.

Xie B., Cheng J., Zhou J., Song W., Liu J. and Cen K. (2008). Production of hydrogen and methane from potatoes by two-phase anaerobic fermentation. *Bioresource Technology*. 99:5942-5946.

Yang P, Zhang R, McGarvey J. A. and Benemann J. R. (2007). Biohydrogen production from cheese processing wastewater by anaerobic fermentation using mixed microbial communities. *International Journal of Hydrogen energy*. 32:4761-4771.

Yokoi H., Tokushige T., Hirose J., Harashi S. and Takasaki Y. (1998). H2 production from starch by a mixed culture of *Clostridium butyricum* and *Enterobactor aerogenes. Biotechnology Letters*. 20:143-147.

Zhanga Z. P., Showa K. Y, Tay J. H., Liang D. T., Lee D. J. and Jiang W. J. (2006). Effect of hydraulic retention time on biohydrogen production and anaerobic microbial community. *Process Biochemistry*. 41:2118-2123.

APPENDIX A

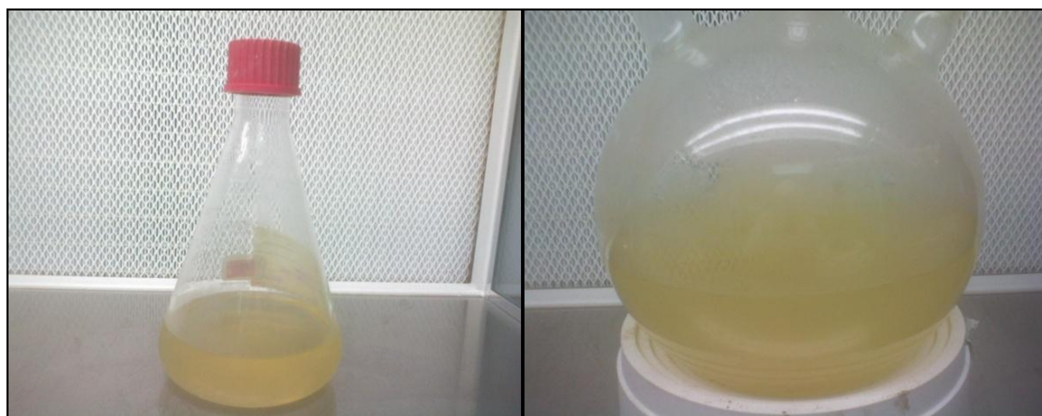
Appendix A.1: UV-Visible Single Beam Spectrophotometer (Model U-1800)

Appendix A.2: Shaking Water Bath (Model BS-21)

Appendix A.3: Double Stack Shaking Incubator Infors

Appendix A.4.: Laminar Air Flow Cabinet (Model AHC-4A1)

APPENDIX B



Appendix B.1: Trial 10 Broth Culture

Appendix B.2: Trial 11 Broth Culture

Appendix B.3: Trial 12 Broth Culture

Appendix B.4: Trial 13 Broth Culture

Appendix B.5: Trial 14 broth culture