

ANAEROBIC DIGESTION OF CRUDE GLYCEROL FOR  
BIOHYDROGEN PRODUCTION

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ANAEROBIC DIGESTION OF CRUDE GLYCEROL FOR  
BIOHYDROGEN PRODUCTION

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*To my dearest late father*

*Ahmad Bin Salim*

*May Allah Blessed You to Jannah*

*Special dedication to*

*My mother Che Kalthom Hassan*

*my father in-law Mr Haron Omar, and my mother in-law Rokiah Din*

*And specifically my beloved and lovely wife*

*Roslindawati Haron.*

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## ABSTRACT

Biodiesel has become one of the main interests for the diesel replacement because it is renewable, and can be produced from various sources. However, the rapid acceleration of biodiesel production consequently contributes towards the mass amount of generation of biodiesel by-product; the waste crude glycerol (CG) which consisted high amount of impurities including the heavy metal that may lead to environmental issues. This has raised the concern and also the interest for the researchers and industries to look for the best solution in managing the excessive crude glycerol. This is included to convert the CG into valuable products, chemically or biologically. Anaerobic digestion process for biohydrogen production using biodiesel waste or crude glycerol as sole substrate seems to have a great potential to be explored. Thus, this study used batch anaerobic fermentation approached using biodiesel wastewater sludge containing indigenous mixed bacteria to produce biohydrogen using CG as the main substrate, and to assess the performance of biohydrogen production under different pH, temperature, incubation time, and initial glycerol concentration. The experiments conducted with the biodiesel wastewater sludge and CG sample were collected from Vance Bioenergy Sdn. Bhd. and Carotino Sdn. Bhd, respectively. The results of the preliminary study using both pure and crude glycerol showed that the mixed culture favours crude glycerol than pure glycerol. There was no methane gas generated throughout this study based on the gas chromatography analysis. Thus, the crude glycerol is further used in this study. Results on the effect of various parameters showed that the mixed culture has successfully converted and utilized the CG up to 99% with hydrogen yield (HY) of 1.05 mol H<sub>2</sub>/mol glycerol utilized with 10 g/l concentration of CG at 48 hours with 37°C. The optimization study showed that, the most significant parameter that influenced the high HY were the pH and temperature. The result of HY at 1.0 – 1.26 mol H<sub>2</sub>/mol glycerol could be achieved under the pH, incubation time, and temperature range were within 7.0 – 9.0, 30 - 55 hours, and 31 – 38°C respectively.

## ABSTRAK

Biodiesel telah menjadi salah satu kepentingan utama dalam menggantikan diesel kerana ia boleh diperbaharui dan boleh dihasilkan daripada pelbagai sumber. Walaubagaimanapun, pengeluaran biodiesel yang terlalu giat akhirnya menyumbang kepada penjanaaan besar-besaran produk sampingan biodiesel; gliserol mentah (CG) yang mengandungi bendasing yang tinggi termasuk logam berat yang boleh menyebabkan isu-isu alam sekitar. Ia menimbulkan kebimbangan dan juga minat bagi penyelidik dan pemain industry untuk mencari penyelesaian terbaik di dalam menangani lebihan gliserol mentah tersebut. Ini termasuklah menukarkan CG kepada produk bernilai, secara kimia atau biologi. Proses penguraian anaerobik untuk pengeluaran biohydrogen menggunakan sisa biodiesel atau gliserol mentah sebagai substrat tunggal dilihat mempunyai potensi yang besar untuk diterokai. Oleh itu, kajian ini menggunakan pendekatan fermentasi anaerobik kelompok untuk menghasilkan biohidrogen menggunakan enap cemar air sisa loji biodiesel mengandungi bacteria campuran asli untuk menghasilkan biohidrogen menggunakan CG sebagai substrat utama dan juga menilai prestasi pengeluaran biohidrogen di bawah keadaan pH, suhu, masa pengeraman dan kepekatan awal CG yang berbeza-beza. Eksperimen dijalankan dengan masing-masing sampel enap cemar air sisa biodiesel dan CG diambil dari Vance Bioenergy Sdn. Bhd. dan Carotino Sdn. Bhd. Keputusan mengikut kajian awal yang menggunakan gliserol tulen dan gliserol mentah menunjukkan bahawa kultur campuran lebih sesuai menggunakan gliserol mentah berbanding gliserol tulen. Tiada gas metana dihasilkan sepanjang kajian ini berdasarkan kepada analisis kromatografi gas. Maka, gliserol mentah terus digunakan selanjutnya di dalam kajian ini. Keputusan daripada kesan pelbagai parameter menunjukkan kultur campuran telah berjaya menukarkan dan menggunakan CG sehingga 99% dengan hasil hydrogen 1.05 mol H<sub>2</sub>/mol gliserol digunakan dengan kepekatan 10 g/l pada 48 jam pengeraman. Kajian pengoptimuman menunjukkan, parameter paling signifikan mempengaruhi HY yang tinggi adalah pH dan suhu. Hasil HY pada 1.0 – 1.26 mol H<sub>2</sub>/mol gliserol dapat dicapai dengan masing-masing julat pH, masa pengeraman, dan suhu pada 7.0 – 9.0, 30 – 55 jam, dan 31 - 38°C.

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**LIST OF ABBREVIATIONS**

1,2-PDO	-	1,2-Propanediol
1,3-PDO	-	1,3-Propanediol
AD	-	Anaerobic Digestion
ANOVA	-	Analysis of Variance
ASBR	-	Anaerobic sequencing batch reactors
ATP	-	Adenosine Triphosphate
CG	-	Crude Glycerol
COD	-	Chemical Oxygen Demand
DoE	-	Design of Experiment
DF	-	Degree of Freedom
DHA	-	Dihydroxyacetone
DHAP	-	Dihydroxyacetone phosphate
DHAK	-	Dihydroxyacetone kinase
FAME	-	Fatty acid methyl ester
FFA	-	Free fatty acid
FHL	-	Formate hydrogen lyase
g/L	-	gram per liter
GC-FID	-	Gas chromatography – flame ionization detector
GC-TCD	-	Gas chromatography – thermal conductive detector
HPLC	-	High performance liquid chromatography
HY	-	Hydrogen Yield
kJ/g	-	kilojoule per gram
MONG	-	Matter Organic Non-Glycerin
MS	-	Mean Square
mL	-	Mililiter

mmol/L/h	-	millimole per liter per hour
OD	-	Optical Density
PG	-	Pure Glycerol
rpm	-	Rotation per minute
RSM	-	Response Surface Methodology
SS	-	Sum of Square
sp	-	Species
WCO	-	Waste cooking oil
VFA	-	volatile fatty acid

**LIST OF SYMBOLS**

%	-	Percent
atm	-	atmosphere
°C	-	degree Celcius
K	-	Kelvin
e <sup>-</sup>	-	Electron
n	-	Mol of gas
P	-	Pressure of gas
V	-	Volume of gas
R	-	Gas constant
T	-	Temperature



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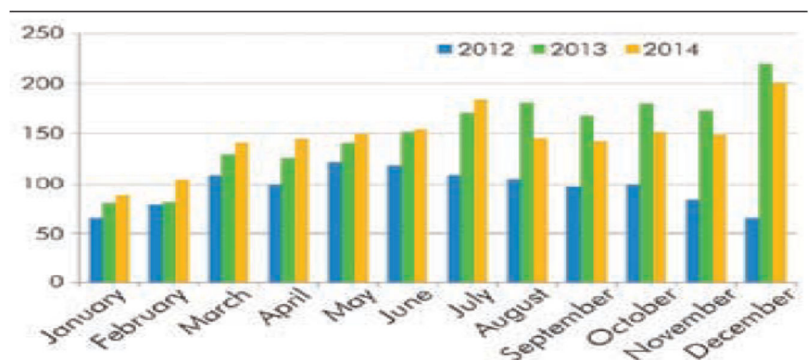
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## CHAPTER 1

### INTRODUCTION

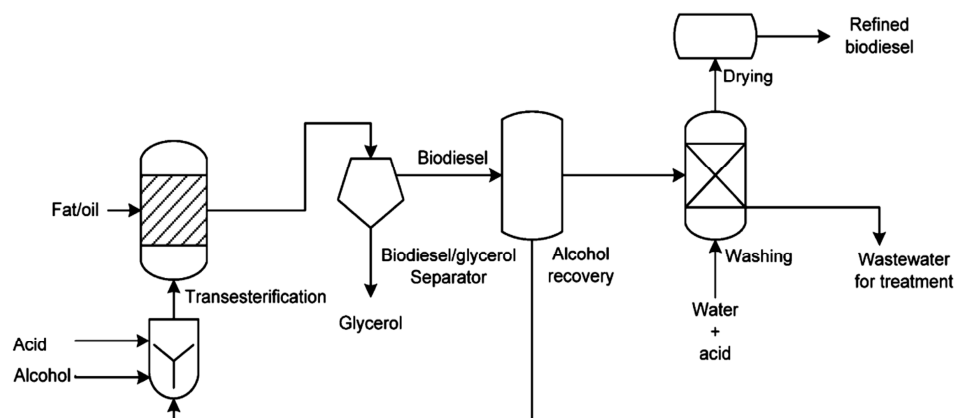
#### 1.1 Research Background

The surge of biodiesel production around the globe has significantly shown United State (US) and Europe as its major producer (Park, 2008, Kotrba, 2015). Figure 1.1 displays the production of biomass-based biodiesel in the US alone with an amount of 200 million gallons (757 million liters) in 2014 (Kotrba, 2015). Kotrba (2015) also highlighted that Germany alone has produced approximately 507 million gallons (1919 million liters) of biodiesel. As predicted by Deloitte (2015), the biodiesel amount is forecasted to increase to 1900 barrels in the year 2020.

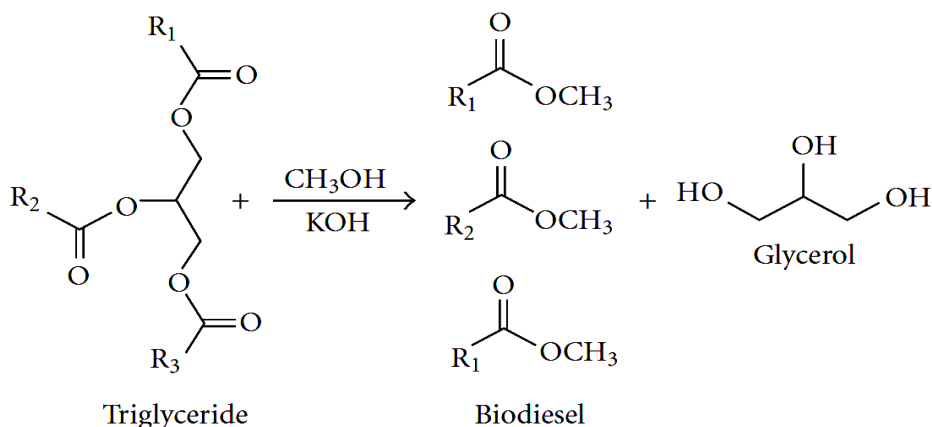


**Figure 1.1** Biodiesel production 2012 – 2014 (million gallons) (Kotrba, 2015)

The general outline of biodiesel production scheme indicating significant processes is shown in Figure 1.2. More importantly, the figure provides an overview of the significant part for crude glycerol generation. In biodiesel production, vegetable oils (corn, cottonseed, crambe, peanut, rapeseed, soybean, sunflower, palm oil, coconut and animal fats (lard and tallow) are the most main commercial ingredients used (Ma and Hanna, 1999; Van Gerpen, 2005; Marchetti *et al.*, 2007; Park, 2008; Borugadda and Goud, 2012; Bergmann *et al.*, 2013). However, these commercial feedstocks are expensive and some are eligible feed (Yaakob *et al.*, 2013). Thus, further advancement in biodiesel production then explored the potential of using used-cooking oil as an alternative to highly priced animal fats and fresh vegetable oils (Siles *et al.*, 2010; Wan Omar and Amin, 2011; Zhang *et al.*, 2003). In general, biodiesel is produced via transesterification process (Figure 1.3), that generates its by-product—crude glycerol (CG). Overall, approximately 10 kg of CG was generated from out of 100 kilogram biodiesel produced from most of the biodiesel plants (Sarma 2013; Santibanez, 2011; Chi *et al.*, 2007). In Malaysia, the abundantly generated CG waste is classified as waste under Schedule S181 of the Malaysia Environmental Regulation, and sealed in drums prior to disposal at landfills (Sakinah, 2011). This scheduled disposal is costly for the collection to ensure the crude glycerol is transferred away from the biodiesel plants for disposal and incineration process (Ayoub and Abdullah, 2012)



**Figure 1.2** Process flow of biodiesel production via conventional transesterification process (Daud *et al.*, 2014)



**Figure 1.3** Transesterification process produce glycerol as main by-products via base catalytic reaction with methanol (Kolesárová *et al.*, 2011; da Silva *et al.*, 2009)

CG contains heavy metals and other contaminants (residues from transesterification process). Methanol, soap, oils, salts and solid organic materials were among contaminants reported in CG (Kumar *et al.*, 2015). The impurities were complied with those reported by Yang *et al.* (2012) except they added catalysts, non-glycerol organic matter, and water impurities in the list of CG contaminants. These contaminants if not treated properly can harm the environment and thus will be costly to treat (Fernando *et al.*, 2007). Some treatment process will be affected by the contaminants, as in Sulaiman *et al.* (2009) and Yang *et al.* (2012), who reported CG with a high concentration of NaCl can cause the biological treatment process to be ineffective. A common practice to destroy CG presently is by incineration, but this method leads to the production of primary green house gases like nitrogen oxide and carbon dioxide (CO<sub>2</sub>) (Gholami *et al.*, 2014; Markov *et al.*, 2011). Thus, an urgent requirement for sound management of CG-containing wastewater along with the potential of CG for biogas generation has generates interest on anaerobic treatment process (Siles *et al.*, 2010, Viana, 2012 and Hutnan *et al.*, 2013).

In recent years, most researchers focus on application of CG and pure glycerol for the production of value-added products (1,3-propanediol, citric acid, erythritol, polyhydroxyalkanoates, phytase, fumaric acid, fungal protein and butanol, butanol,)

using mixed and pure cultures of microorganisms (bacteria and fungus) as summarized in Table 1.1

**Table 1.1** : Application of CG and PG for value added product

<b>Value added product</b>	<b>Glycerol type</b>	<b>References</b>
<b>Hydrogen</b>	Crude glycerol	(Sarma <i>et al.</i> , 2013)
<b>Hydrogen</b>	Crude glycerol	(Ghosh <i>et al.</i> , 2012)
<b>Methane</b>	Crude glycerol	(Siles López <i>et al.</i> , 2009)
<b>Methane</b>	Crude glycerol	(Hutnan <i>et al.</i> , 2013)
<b>Ethanol</b>	Pure glycerol	(Legendre <i>et al.</i> , 2009)
<b>Dihydroxyacetone</b>	Crude glycerol	(Liu <i>et al.</i> , 2013)
<b>Poly 3-hydroxybutyrate and bioethanol</b>	Crude glycerol	(Shah <i>et al.</i> , 2014)
<b>Biogas</b>	Crude glycerol	(Nuchdang and Phalakornkule, 2012)
<b>Biohydrogen</b>	Crude glycerol	(Costa <i>et al.</i> , 2011)
<b>Butanol</b>	Crude glycerol	(Gallardo <i>et al.</i> , 2014)
<b>1,3-propanediol and gellan</b>	Crude glycerol	(Raghunandan, 2013)
<b>n-butanol</b>	Crude glycerol	(Khanna <i>et al.</i> , 2013)
<b>Docosaehaenoic acid</b>	Crude glycerol	(Chi <i>et al.</i> , 2007)
<b>Lipid</b>	Crude glycerol	(Liang <i>et al.</i> , 2010)
<b>1,3-propanediol and ethanol</b>	Crude glycerol	(Rossi <i>et al.</i> , 2012)
<b>Glyceric acid and hydrogen</b>	Crude glycerol	(Kondamudi <i>et al.</i> , 2012)
<b>Omega-3 and carotenoids</b>	Pure glycerol	(Adarsha Gupta <i>et al.</i> , 2013)
<b>Hydrogen, ethanol, and diols</b>	Crude glycerol	(Wu <i>et al.</i> , 2011)
<b>Succinic acid</b>	Crude glycerol	(Vlysidis <i>et al.</i> , 2011)

A quite similar scenario was also developed for biodegradation of banana peel wastewater using CG or pure glycerol (PG) as co-substrate under buffered (0.1 NaHCO<sub>3</sub>) and unbuffered fermentation to observe the potential of biomethane production (Housagul *et al.*, 2014). From the analysis, they found that addition of buffer in both mixtures helps in improving the content, production potential, lag phase period, and yield of methane. They also found that glycerol concentration can influence the rate and yield of the methane produced.

According to Ma *et al.* (2008) and Wohlgemut (2011), using CG as co-substrate in anaerobic digestion gives the advantage to some other wastes. This is because, CG is degradable, contains a high level of COD, and can be stored for long periods before being used in biogas production. Nevertheless, co-digestion has weaknesses too; in terms of cost, as well as the risks and process itself. According to Astals *et al.* (2011), transporting CG or other co-substrates to the anaerobic plant can be costly. The transporting act can also lead to the risk of spreading poisonous substances. In terms of quality, co-digestion can lower the digestate quality, change the digestion behaviour, and need a proper design so the ratio between the main- and co-substrate will not affect the digestive medium.

Despite a number of studies on anaerobic co-digestion of CG and digestion of CG using pure cultures, there have been few studies on using CG as sole substrate by mixed cultures. Hutnan *et al.* (2013) for example, investigates the use of CG as the main substrate by mixed cultures from biodiesel sludge as biomethane producer. They used two types of CG in their study: acidulated CG (80% of glycerol) and untreated CG (55% of glycerol). The process using acidulated CG, was enhanced by the addition of washing water (or biodiesel wastewater). Their findings showed no significant difference between both process; they obtained about 0.328L/g chemical oxygen demand (COD) for non-acidulated CG and 0.345L/g COD for acidulated CG. They also proved that the use of CG and mixed cultures in anaerobic digestion was promising in producing biogas.

Another study by Nuchdang and Phalakornkule (2012) on the used of CG as the single substrate has been studied in a continuous digester. However, studies on using raw CG with locally isolated indigenous microorganisms in biohydrogen production is still at infancy state. Thus, this study was performed by applying batch anaerobic digestion process to transform the biodiesel waste by-product or crude glycerol to biohydrogen using locally isolated mixed microorganism.

## 1.2 Problem Statement

Research on the application of CG as the main substrate in biogas production that has been done so far mostly used pure cultures (Abdeshahian *et al.*, 2014; Chen *et al.*, 2008; Chookaew *et al.*, 2011; Chookaew *et al.*, 2014) as producers. According to Elsharnouby *et al.* (2013), pure cultures offer more insights on metabolic shift and conditions that can promote high hydrogen yield and production rate. However, pure cultures are not resilient enough with the change of environment, thus need to be adapted first. In addition, pure culture must also be steriled enough to avoid contamination. Secondly, the most CG-related biogas production research was done using the CG as co-substrate instead of main substrate (Mata-Alvarez *et al.*, 2014). Research by Mata-Alvarez *et al.* (2014) critically discussed the function of CG as a co-digestion substrate for treatment of other waste such as olive mill waste (OMW), animal manure (pig, cow, poultry, goat, horse, etc.) organic fraction-municipal solid waste (OFMSW), and cheese whey. Another work by Kullavanijaya and Thongduang (2012) also used anaerobic digestion to treat cassava wastewater with biodiesel waste as the supplement or co-substrate it to enhance the production of biogas and methane, as well as for the chemical oxygen demand (COD) removal.

Crude glycerol (CG) from biodiesel waste contain impurities that can harm environment and ecosystem if been disposed of without proper management. The existing approaches in handling this problem are by incineration but this approach generates greenhouse gases. In order to minimize the generation of the GHG production, a better and sustainable treatment of crude glycerol is vital for generation of future renewable energy, the biohydrogen. Since research on using crude glycerol as the sole carbon source for biohydrogen production still lacking, the urge for its potential is very promising. Even though the studies by Hutnan *et al.*, 2009, 2011, and 2013 have claimed that there is potential in using crude glycerol as the main carbon source in their study, their main focus was specifically on biomethane production. Additionally, their seeding inoculums were originating from suspended sludge taken from municipal wastewater treatment plant which is totally not an adapted indigenous source of inoculums for crude glycerol fermentation. Meanwhile,



the study by Sarma *et al.*, 2013 mentioned clearly that the inoculum used was a commercially known single culture of *Enterobacter aerogens* NRRL B-407 for the fermentation of their crude glycerol.

Thus, in this study, to differentiate between the studies by other researchers mentioned earlier, the anaerobic digestion process is performed totally using crude glycerol as the main source of carbon for the biohydrogen production using sludge originating from the biodiesel wastewater treatment plant, which potentially well adapted with the existing crude glycerol element.

### **1.3 Research Objectives**

This work is proposed to utilize the waste crude glycerol in the anaerobic treatment process for the potential production of biohydrogen. The main objectives for this work are:

1. To apply batch anaerobic digestion system using the mixed culture of bacteria from biodiesel wastewater sludge to utilize crude glycerol for biohydrogen production.
2. To assess the biohydrogen production from the anaerobic process using local microorganisms under four operating parameters: pH, temperature, incubation time, and initial crude glycerol concentration.

## 1.4 Research Scopes

The scopes of this study involved:

1. The sampling of anaerobic sludge from the biodiesel wastewater treatment plant and crude glycerol from edible oil manufacturer in Pasir Gudang, Johor.
  - i. Characterization of crude glycerol samples
  - ii. Sludge samples preservation to maintain its microbiological content viability
2. Develop an anaerobic digestion for batch fermentation process of the crude glycerol
  - i. Batch anaerobic system setup at 37°C, with initial pH of 7 and 48 hours incubation time.
  - ii. Design of Experiment (DoE) for optimum operational parameters determination: pH, temperature, incubation period, and crude glycerol initial concentration
3. Monitoring the biohydrogen production and percentage of crude glycerol consumption

## 1.5 Research Significance

Crude glycerol cannot be disposed into the environment without any treatment, and the cost of such treatment could be prohibitive. Methanol, one of the impurities in crude glycerol, is known to be a toxic alcohol. It can affect soil microbial flora if crude glycerol is disposed of to the environment. It can contaminate ground waters too. The sodium or potassium hydroxide residue of the

crude glycerol gives it elevated pH, may endanger biotic community if crude glycerol is directly disposed of without neutralization. Moreover, the action of indigenous microorganisms will release the offensive odor that pollutes the atmospheric environment (Sarma *et al.*, 2013). Therefore, to treat crude glycerol before disposal is an economically inefficient and ineffective process for biodiesel industries (Nwachukwu, 2012).

## REFERENCES

- Abdeshahian, P., Kaid, N., Al-shorgani, N., Abdul, A., and Sahaid, M. (2014). The Production of Biohydrogen by a Novel Strain *Clostridium* sp . YM1 in Dark Fermentation Process. *International Journal of Hydrogen Energy*, 39(24), 12524–12531.
- Aquino de Souza, E., Rossi, D. M., and Záchia Ayub, M. A. (2014). Bioconversion of Residual Glycerol from Biodiesel Synthesis into 1,3-propanediol using Immobilized Cells of *Klebsiella pneumoniae* BLh-1. *Renewable Energy*, 72, 253–257.
- Araque, M., Martínez T, L. M., Vargas, J. C., and Roger, A. C. (2011). Hydrogen Production by Glycerol Steam Reforming over Cezrco Fluorite Type Oxides. *Catalysis Today*, 176(1), 352–356.
- Ardi, M. S., Aroua, M. K., and Hashim, N. A. (2015). Progress, Prospect and Challenges in Glycerol Purification Process: A Review. *Renewable and Sustainable Energy Reviews*, 42, 1164–1173.
- Astals, S., Ariso, M., and Galí, A. (2011). Co-Digestion of Pig Manure and Glycerine : Experimental and Modelling Study. *Journal of Environmental Management*, 92(4), 1091–1096.
- Athanasoulia, E., Melidis, P., and Aivasidis, A. (2014). Co-digestion of Sewage Sludge and Crude Glycerol from Biodiesel Production. *Renewable Energy*, 62, 73–78.
- Ayoub, M., and Abdullah, A. Z. (2012). Critical Review on the Current Scenario and Significance of Crude Glycerol Resulting from Biodiesel Industry towards More Sustainable Renewable Energy Industry. *Renewable and Sustainable Energy Reviews*, 16(5), 2671–2686.

- Baba, Y., Tada, C., Watanabe, R., Fukuda, Y., Chida, N., and Nakai, Y. (2013). Anaerobic Digestion of Crude Glycerol from Biodiesel Manufacturing using a Large-Scale Pilot Plant: Methane Production and Application of Digested Sludge as Fertilizer. *Bioresource Technology*, 140, 342–348.
- Bailey JE and Ollis DF. 1986. *Biochemical Engineering Fundamentals*, 2nd edn. McGraw-Hill, New York.
- Bergmann, J. C., Tupinambá, D. D., Costa, O. Y. a, Almeida, J. R. M., Barreto, C. C., and Quirino, B. F. (2013). Biodiesel Production in Brazil and Alternative Biomass Feedstocks. *Renewable and Sustainable Energy Reviews*, 21, 411–420.
- Borugadda, V. B., and Goud, V. V. (2012). Biodiesel Production from Renewable Feedstocks: Status and Opportunities. *Renewable and Sustainable Energy Reviews*, 16(7), 4763–4784.
- Cerqueira, S., Liebensteiner, M. G., and Cristiane, L. (2015). Crude Glycerol as a Substrate for Sulfate-reducing Bacteria from a Mature Oil Field and its Potential Impact on Souring. *Journal of Petroleum Science and Technology* 5(1), 1–9.
- Chatterjee, I., Somerville, G. A., Heilmann, C., Sahl, H. G., Maurer, H. H., and Herrmann, M. (2006). Very Low Ethanol Concentrations Affect the Viability And Growth Recovery in Post-Stationary-Phase *Staphylococcus Aureus* Populations. *Appl Environ Microbiol*, 72(4), 2627–2636.
- Chen, S. D., Lee, K. S., Lo, Y. C., Chen, W. M., Wu, J. F., Lin, C. Y., and Chang, J. S. (2008). Batch and Continuous Biohydrogen Production from Starch Hydrolysate by *Clostridium* species. *International Journal of Hydrogen Energy*, 33(7), 1803–1812.
- Cheong, D.-Y., and Hansen, C. L. (2006). Bacterial Stress Enrichment Enhances Anaerobic Hydrogen Production in Cattle Manure Sludge. *Applied Microbiology and Biotechnology*, 72(4), 635–643.
- Chi, Z., Pyle, D., Wen, Z., Frear, C., and Chen, S. (2007). A Laboratory Study of Producing Docosahexaenoic Acid from Biodiesel-Waste Glycerol by Microalgal Fermentation. *Process Biochemistry*, 42(11), 1537–1545.
- Chookaew, T., O-thong, S., and Prasertsan, P. (2011). Effect of Culture Conditions on Biohydrogen Production from Crude Glycerol of Biodiesel Plant by

- Thermotolerant *Klebsiella sp.* TR17. In *TIChE International Conference 2011* (p. 1). November 10-11, 2011. Songkhla Thailand.
- Chookaew, T., O-Thong, S., and Prasertsan, P. (2012). Fermentative Production of Hydrogen and Soluble Metabolites from Crude Glycerol of Biodiesel Plant by the Newly Isolated Thermotolerant *Klebsiella pneumoniae* TR17. *International Journal of Hydrogen Energy*, 37(18), 13314–13322.
- Chookaew, T., O-Thong, S., and Prasertsan, P. (2014). Biohydrogen Production from Crude Glycerol by Immobilized *Klebsiella Sp.* TR17 in a UASB Reactor and Bacterial Quantification Under Non-Sterile Conditions. *International Journal of Hydrogen Energy*, 39(18), 9580–9587.
- Chookaew, T., Prasertsan, P., and Ren, Z. J. (2014). Two-stage Conversion of Crude Glycerol to Energy using Dark Fermentation Linked with Microbial Fuel Cell or Microbial Electrolysis Cell. *New Biotechnology*, 31(2), 179–184.
- Clomburg, J. M., and Gonzalez, R. (2013). Anaerobic Fermentation of Glycerol: A Platform for Renewable Fuels and Chemicals. *Trends in Biotechnology*, 31(1), 20–28.
- Collins, G., and Er, T. H. (2010). Anaerobic Digestion of Agricultural Residues, *International Journal of Green Energy*, 259–279.
- Costa, J. B., Rossi, D. M., De Souza, E. a, Samios, D., Bregalda, F., do Carmo Ruaro Peralba, M., Ayub, M. A. Z. (2011). The Optimization of Biohydrogen Production by Bacteria Using Residual Glycerol from Biodiesel Synthesis. *Journal of Environmental Science and Health. Part A, Toxic/hazardous Substances and Environmental Engineering*, 46(13), 1461–8.
- Daud, N. M., Sheikh Abdullah, S. R., Abu Hasan, H., and Yaakob, Z. (2014). Production of Biodiesel and Its Wastewater Treatment Technologies: A Review. *Process Safety and Environmental Protection*, 94(October), 487–508.
- de Mes, T. Z. D., Stams, A. J. M., Reith, J. H., and Zeeman, G. (2003). Methane Production by Anaerobic Digestion of Wastewater and Solid. In *Bio-Methane and Bio-Hydrogen, Status and Perspectives of Biological Methane and Hydrogen Production*, 58 –102.
- Deloitte. (2015). *The Feedstocks Prism Unveiling Value in Volatile and Complex Petrochemicals*,  
<http://www2.deloitte.com/content/dam/Deloitte/cn/Documents/manufacturing/>

deloitte-cn-mfg-chem-feedstocksprism-en-150907.pdf Accessed February 2015).

- Duane T. Johnson, K. A. T. (2009). The Glycerin Glut: Options for the Value-Added Conversion of Crude Glycerol Resulting from Biodiesel Production. *Environmental Progress*, 28(3), 404–409.
- Elbeshbishy, E. (2011). *Enhancement of Biohydrogen and Biomethane Production from Wastes Using Ultrasonication*.
- Elsharnouby, O., Hafez, H., Nakhla, G., and El Nagggar, M. H. (2013). A Critical Literature Review on Biohydrogen Production by Pure Cultures. *International Journal of Hydrogen Energy*, 38(12), 4945–4966.
- Fangkum, A., and Reungsang, A. (2011). Biohydrogen Production From Mixed Xylose/Arabinose at Thermophilic Temperature by Anaerobic Mixed Cultures in Elephant Dung. *International Journal of Hydrogen Energy*, 36(21), 13928–13938.
- Feng, X., Ding, Y., Xian, M., Xu, X., Zhang, R., and Zhao, G. (2014). Production of Optically Pure D-Lactate from Glycerol by Engineered *Klebsiella pneumoniae* Strain. *Bioresource Technology*, 172C, 269–275.
- Fernando, S., Adhikari, S., Kota, K., and Bandi, R. (2007). Glycerol Based Automotive Fuels from Future Biorefineries. *Fuel*, 86(17-18), 2806–2809.
- Gadhamshetty, V., Johnson, D., Nirmalakhandan, N., Smith, G., and Deng, S. (2009). Feasibility of Biohydrogen Production at Low Temperatures in Unbuffered Reactors. *International Journal of Hydrogen Energy*, 34(3), 1233–1243.
- Gallardo, R., Alves, M., and Rodrigues, L. R. (2014). Modulation of Crude Glycerol Fermentation by *Clostridium Pasteurianum* DSM 525 Towards the Production of Butanol. *Biomass and Bioenergy*, 71, 134–143.
- Ghimire, A., Frunzo, L., Pontoni, L., Lens, P. N. L., Esposito, G., and Pirozzi, F. (2015). Dark Fermentation Of Complex Waste Biomass for Biohydrogen Production by Pretreated Thermophilic Anaerobic Digestate. *Journal of Environmental Management*, 152, 43–48.
- Gholami, Z., Abdullah, A. Z., and Lee, K.-T. (2014). Dealing with the Surplus of Glycerol Production from Biodiesel Industry Through Catalytic Upgrading to Polyglycerols and Other Value-Added Products. *Renewable and Sustainable Energy Reviews*, 39, 327–341.

- Ghosh, D., Sobro, I. F., and Hallenbeck, P. C. (2012). Stoichiometric Conversion of Biodiesel Derived Crude Glycerol to Hydrogen: Response Surface Methodology Study of the Effects of Light Intensity and Crude Glycerol and Glutamate Concentration. *Bioresource Technology*, 106, 154–160.
- Gonçalves, M. R., Costa, J. C., Pereira, M. A., Abreu, A. A., and Alves, M. M. (2014). On The Independence of Hydrogen Production from Methanogenic Suppressor In Olive Mill Wastewater. *International Journal of Hydrogen Energy*, 39(12), 6402–6406.
- Gupta, A., Singh, D., Barrow, C. J., and Puri, M. (2013). Exploring Potential Use Of Australian Thraustochytrids for the Bioconversion of Glycerol to Omega-3 and Carotenoids Production. *Biochemical Engineering Journal*, 78, 11–17.
- Haaland P.D. (1989), Experimental design in biotechnology. Marcel Dekker, INC., New York.
- Hallenbeck, P. C. (2009). Fermentative Hydrogen Production: Principles, Progress, and Prognosis. *International Journal of Hydrogen Energy*, 34(17), 7379–7389.
- Hansen, C. F., Hernandez, A., Mullan, B. P., Moore, K., Trezona-Murray, M., King, R. H., and Pluske, J. R. (2009). A Chemical Analysis of Samples of Crude Glycerol from the Production of Biodiesel in Australia, and the Effects of Feeding Crude Glycerol to Growing-Finishing Pigs on Performance, Plasma Metabolites and Meat Quality at Slaughter. *Animal Production Science*, 49, 154–161.
- Housagul, S., Sirisukpoka, U., Boonyawanich, S., and Pisutpaisal, N. (2014). Biomethane Production from Co-digestion of Banana Peel and Waste Glycerol. *Energy Procedia*, 61, 2219–2223.
- Hu, S., Luo, X., Wan, C., and Li, Y. (2012). Characterization of Crude Glycerol from Biodiesel Plants. *Journal of Agricultural and Food Chemistry*, 60(23), 5915–5921.
- Huffer, S., Clark, M. E., Ning, J. C., Blanch, H. W., and Clark, D. S. (2011). Role of Alcohols in Growth, Lipid Composition, and Membrane Fluidity of Yeasts, Bacteria, and Archaea. *Applied and Environmental Microbiology*, 77(18), 6400–6408.



- Hutnan, M., Kolesárová, N., and Bodík, I. (2013). Anaerobic Digestion of Crude Glycerol as Sole Substrate in Mixed Reactor. *Environmental Technology*, 2179-2187.
- Hutnan, M., Kolesárová, N., Bodík, I., Spalková, V., and Lazor, M. (2009). Possibilities Of Anaerobic Treatment of Crude Glycerol from Biodiesel Production. *36th International Conference of SSCHE*, 156 p.
- Ito, T., Nakashimada, Y., Senba, K., Matsui, T., and Nishio, N. (2005). Hydrogen and Ethanol Production from Glycerol-Containing Wastes Discharged after Biodiesel Manufacturing Process. *Journal of Bioscience and Bioengineering*, 100(3),
- Jain, A. (2009). Biohydrogen Production from Cull Peach Medium by Hyperthermophilic Bacterium, *Thermotoga neapolitana*. Analysis.
- Jensen, T. O., Kvist, T., Mikkelsen, M. J., Christensen, P. V., and Westermann, P. (2012). Fermentation of Crude Glycerol from Biodiesel Production by *Clostridium pasteurianum*. *Journal of Industrial Microbiology and Biotechnology*, 39(5), 709–17.
- Johari, A., Nyakuma, B. B., Mohd Nor, S. H., Mat, R., Hashim, H., Ahmad, A., ... Tuan Abdullah, T. A. (2015). The Challenges and Prospects of Palm Oil Based Biodiesel in Malaysia. *Energy*, 81, 255–261.
- Khanna, S., Goyal, A., and Moholkar, V. S. (2013). Production of n-butanol from Biodiesel derived Crude Glycerol using *Clostridium pasteurianum* Immobilized on Amberlite. *Fuel*, 112, 557–561.
- Kolesárová, N., Hutnan, M., Bodík, I., and Špalková, V. (2011). Utilization of Biodiesel By-products for Biogas Production. *Journal of Biomedicine and Biotechnology*, 2011.
- Kondamudi, N., Misra, M., Banerjee, S., Mohapatra, S., and Mohapatra, S. (2012). Simultaneous Production of Glyceric Acid and Hydrogen from the Photooxidation of Crude Glycerol using TiSi 2. *Applied Catalysis B: Environmental*, 126, 180–185.
- Kotrba, R. (2015). *Biodiesel Magazine. Practice*, 2–3. Retrieved from <http://www.biodieselmagazine.com/articles/341782/german-biodiesel-exports-imports-up-in-2014> on 24th December 2015
- Kullavanijaya, P., and Thongduang, P. (2012). Enhanced Biogas Production in Anaerobic Digestion of Cassava Wastewater Through Supplementation of

- Biodiesel Waste as Co-Substrate. *International Journal of Renewable Energy Research*, 2(3), 510–515.
- Kumar, P., Sharma, R., Ray, S., Mehariya, S., Patel, S. K. S., Lee, J.-K., and Kalia, V. C. (2015). Dark Fermentative Bioconversion of Glycerol to Hydrogen by *Bacillus thuringiensis*. *Bioresource Technology*, 182, 383–8.
- Lee, K., Chen, S., and Nakhla, G. (2013). *Chapter 9. Biological Hydrogen Production: Dark Fermentation*. In A. S. S. A. Sherif, D. Yogi Goswami, E. K. Lee Stefanakos (Ed.), *Handbook of Hydrogen Energy*.
- Legendre, C., Logan, E., Mendel, J., and Seedial, T. (2009). Anaerobic Fermentation of Glycerol to Ethanol. Senior Design Report, University of Pennsylvania.
- Li, C., Lesnik, K. L., and Liu, H. (2013). Microbial Conversion of Waste Glycerol from Biodiesel Production into Value-Added Products. *Energies*, 6(9), 4739–4768.
- Liang, Y., Cui, Y., Trushenski, J., and Blackburn, J. W. (2010). Converting Crude Glycerol Derived from Yellow Grease to Lipids Through Yeast Fermentation. *Bioresource Technology*, 101(19), 7581–7586.
- Liang, Y., Sarkany, N., Cui, Y., and Blackburn, J. W. (2010). Batch Stage Study of Lipid Production from Crude Glycerol Derived from Yellow Grease or Animal Fats Through Microalgal Fermentation. *Bioresource Technology*, 101(17), 6745–6750.
- Liu, Y. P., Sun, Y., Tan, C., Li, H., Zheng, X. J., Jin, K. Q., and Wang, G. (2013). Efficient Production of Dihydroxyacetone from Biodiesel-Derived Crude Glycerol by Newly Isolated *Gluconobacter frateurii*. *Bioresource Technology*, 142, 384–389.
- Liu, Y., Koh, C. M. J., and Ji, L. (2011). Bioconversion of Crude Glycerol to Glycolipids in *Ustilago maydis*. *Bioresource Technology*, 102(4), 3927–3933.
- Ma, F., and Hanna, M. a. (1999). Biodiesel Production: A Review Journal Series #12109, Agricultural Research Division, Institute of Agriculture and Natural Resources, University of Nebraska–Lincoln.1. *Bioresource Technology*, 70(1), 1–15.
- Mao, C., Feng, Y., Wang, X., and Ren, G. (2015). Review on Research Achievements of Biogas from Anaerobic Digestion. *Renewable and Sustainable Energy Reviews*, 45, 540–555.

- Marchetti, J. M., Miguel, V. U., and Errazu, a. F. (2007). Possible Methods for Biodiesel Production. *Renewable and Sustainable Energy Reviews*, 11(6), 1300–1311.
- Markov, S. A., Averitt, J., and Waldron, B. (2011). Bioreactor for Glycerol Conversion into H<sub>2</sub> by Bacterium *Enterobacter aerogenes*. *International Journal of Hydrogen Energy*, 36(1), 262–266.
- Maru, B. T. (2013). *Sustainable Production of Hydrogen and Chemical Commodities from Biodiesel Waste Crude Glycerol and Cellulose by Biological and Catalytic Processes*. Universitat Rovira i Virgili.
- Mata-Alvarez, J., Dosta, J., Romero-Güiza, M. S., Fonoll, X., Peces, M., and Astals, S. (2014). A Critical Review on Anaerobic Co-Digestion Achievements between 2010 and 2013. *Renewable and Sustainable Energy Reviews*, 36, 412–427.
- McNeil, T. (2005). *Colorado Agriculture IOF Technology Assessments : Anaerobic Digestion*. Program, (September).
- Moita, R., Freches, A., and Lemos, P. C. (2014). Crude Glycerol as Feedstock for Polyhydroxyalkanoates Production by Mixed Microbial Cultures. *Water Research*, 58, 9–20.
- Moon, C., Jang, S., Yun, Y.-M., Lee, M.-K., Kim, D.-H., Kang, W.-S., Kim, M.-S. (2014). Effect of the Accuracy of pH Control on Hydrogen Fermentation. *Bioresource Technology*, 179, 595–601.
- Nartker, S., Ammerman, M., Aurandt, J., Stogsdil, M., Hayden, O., and Antle, C. (2014). Increasing Biogas Production from Sewage Sludge Anaerobic Co-Digestion Process by Adding Crude Glycerol from Biodiesel Industry. *Waste Management*, 34(12), 2567–2571.
- Nghiem, L. D., Nguyen, T. T., Manassa, P., Fitzgerald, S. K., Dawson, M., and Vierboom, S. (2014). Co-Digestion of Sewage Sludge and Crude Glycerol for On-Demand Biogas Production. *International Biodeterioration and Biodegradation*, 95, 160–166.
- Nitayavardhana, S., and Khanal, S. K. (2011). Biodiesel-Derived Crude Glycerol Bioconversion to Animal Feed: A Sustainable Option for a Biodiesel Refinery. *Bioresource Technology*, 102(10), 5808–14.

- Nuchdang, S., and Phalakornkule, C. (2012). Anaerobic Digestion of Glycerol and Co-Digestion of Glycerol and Pig Manure. *Journal of Environmental Management*, 101, 164–172.
- Nwachukwu, R. E. S. (2012). *Biological Conversion of Glycerol to Ethanol by Enterobacter aerogenes*. North Carolina A and T State University.
- OECD, FAO. (2015). OECD-FAO Agricultural Outlook 2015.
- Pagliaro, M., and Rossi, M. (2008). Glycerol : Properties and Production. In M. R. Mario Pagliaro (Ed.), *RSC Green Chemistry Book Series- The Future of Glycerol: New Uses of a Versatile Raw Material* (2nd ed., pp. 1–18). Royal Society of Chemistry 2015.
- Panpong, K., Srisuwan, G., O-Thong, S., and Kongjan, P. (2014). Anaerobic Co-digestion of Canned Seafood Wastewater with Glycerol Waste for Enhanced Biogas Production. *Energy Procedia*, 52, 328–336.
- Park, S. (2008). *Crude Glycerin for Monogastric Feeds*, Render Magazine August 2008 pg 10-11.
- Patil, J. H., Shetty, V., Hosur, M., and Muralidhara, P. L. (2012). Impact of Incubation Period of Primary Sludge Inoculum on Biomethanation of Water Hyacinth. *Journal of Chemical and Pharmaceutical Research*, 4(3), 1719-1724.
- Pawlicka, J., Kubiak, P., and Kos, A. (2014). Conversion of Glycerol to 1, 3-propanediol by *Citrobacter freundii* and *Hafnia alvei* – Newly Isolated Strains from the Enterobacteriaceae. *New Biotechnology*, 31(5), 20–22.
- Philpott, J. (2011). Bio-hydrogen Production from Glucose Degradation using a Mixed Anaerobic Culture in the Presence of Natural and Synthetic Inhibitors. Master Thesis, University of Windsor.
- Pyle, D. J. (2008). Use of Biodiesel-Derived Crude Glycerol for the Production of Omega-3 Polyunsaturated Fatty Acids by the Microalga *Schizochytrium limacinum*. *Work*, 83.
- Qin, Z., and Li, D. (2012). Hydrogen-production of Hydrogen-producing Bacteria at Different Temperatures in Batch Culture. *Advanced Research Materials*, 515, 1400–1403.
- Raghunandan, K. (2013). Bioconversion Of Biodiesel-derived Crude Glycerol Waste to 1,3-propanediol and Gellan Using Adapted Bacterial Isolates. Master Thesis, Durban University of Technology, 108.

- Razaviarani, V., Buchanan, I. D., Malik, S., and Katalambula, H. (2013). Pilot Scale Anaerobic Co-Digestion of Municipal Wastewater Sludge with Biodiesel Waste Glycerin. *Bioresource Technology*, 133, 206–212.
- Romero Aguilar, M. A., Fdez-Güelfo, L. A., Álvarez-Gallego, C. J., and Romero García, L. I. (2013). Effect of HRT on Hydrogen Production and Organic Matter Solubilization in Acidogenic Anaerobic Digestion of OFMSW. *Chemical Engineering Journal*, 219, 443–449.
- Rossi, D. M., da Costa, J. B., de Souza, E. A., Peralba, M. D. C. R., and Ayub, M. A. Z. (2012). Bioconversion of Residual Glycerol from Biodiesel Synthesis into 1,3-propanediol and Ethanol by Isolated Bacteria from Environmental Consortia. *Renewable Energy*, 39(1), 223–227.
- Sakinah, a M. M. (2011). Treatment of Glycerin Pitch from Biodiesel Production. *International Journal of Chemical and Environmental Engineering*, 2(5), 309–313.
- Sarma, S. J., Dhillon, G. S., Brar, S. K., Le Bihan, Y., Buelna, G., and Verma, M. (2013). Investigation of the Effect of Different Crude Glycerol Components on Hydrogen Production by *Enterobacter aerogenes* NRRL B-407. *Renewable Energy*, 60, 566–571.
- Satpathy, P., Thosar, A., and Rajan, A. P. (2014). Green Technology for Glycerol Waste from Biodiesel Plant. *International Journal of Current Microbiology and Applied Sciences*, 3(1), 730–739.
- Saxena, R. K., Anand, P., Saran, S., and Isar, J. (2009). Microbial Production of 1,3-propanediol: Recent Developments and Emerging Opportunities. *Biotechnology Advances*, 27(6), 895–913.
- Shah, P., Chiu, F.-S., and Lan, J. C.-W. (2014). Aerobic Utilization of Crude Glycerol by Recombinant *Escherichia coli* for Simultaneous Production of Poly 3-hydroxybutyrate and Bioethanol. *Journal of Bioscience and Bioengineering*, 117(3), 343–50.
- Siles López, J. Á., Martín Santos, M. D. L. Á., Chica Pérez, A. F., and Martín Martín, A. (2009). Anaerobic Digestion of Glycerol Derived from Biodiesel Manufacturing. *Bioresource Technology*, 100(23), 5609–5615.
- Siles, J. a., Martín, M. a., Chica, a. F., and Martín, A. (2010). Anaerobic Co-digestion of Glycerol and Wastewater Derived from Biodiesel Manufacturing. *Bioresource Technology*, 101(16), 6315–6321.

- Silvey, L. (2012). *Hydrogen and Syngas Production from Biodiesel Derived Crude Glycerol*. Retrieved from <https://kuscholarworks.ku.edu/dspace/handle/1808/9697> on 13th October 2015
- Sivaramakrishna, D., Sreekanth, D., and Sivaramakrishnan, M. (2014). Effect of System Optimizing Conditions on Biohydrogen Production from Herbal Wastewater by Slaughterhouse Sludge. *International Journal of Hydrogen Energy*, 39(14), 7526–7533.
- Stamatelatou, K., Antonopoulou, G., and Michailides, P. (2014). 15 - Biomethane and Biohydrogen Production via Anaerobic Digestion/Fermentation. In *Advances in Biorefineries* (pp. 476–524). Woodhead Publishing Limited.
- Sulaiman, a., Zakaria, M. R., Hassan, M. a., Shirai, Y., and Busu, Z. (2009). Co-Digestion of Palm Oil Mill Effluent and Refined Glycerin Wash Water for Chemical Oxygen Demand Removal and Methane Production. *American Journal of Environmental Sciences*, 5(5), 639–646.
- Szymanowska-Powalowska, D. (2015). The Effect of High Concentrations of Glycerol on the Growth, Metabolism and Adaptation Capacity of *Clostridium butyricum* DSP1. *Electronic Journal of Biotechnology*, 18(2), 128–133.
- Tian, Z. (2011). Anaerobic Digestion of Biofuel Production Residues. PhD Dissertation, University of Florida.
- Tianfeng, C., Huipeng, L., Hua, Z., and Kejian, L. (2013). Purification of Crude Glycerol from Waste Cooking Oil Based Biodiesel Production by Orthogonal Test Method, *China Petroleum Processing and Petrochemical Technology* 15(1), 48–53.
- Van Gerpen, J. (2005). Biodiesel Processing and Production. *Fuel Processing Technology*, 86(10), 1097–1107.
- Varrone, C., Rosa, S., Fiocchetti, F., Giussani, B., Izzo, G., Massini, G., Wang, A. (2013). Enrichment of Activated Sludge for Enhanced Hydrogen Production from Crude Glycerol. *International Journal of Hydrogen Energy*, 38(3), 1319–1331.
- Venkata Mohan, S., and Pandey, A. (2013). Biohydrogen Production: An Introduction. In *Biohydrogen* (pp. 1–24). Elsevier B.V.

- Verhoef, S., Gao, N., Ruijssenaars, H. J., and de Winde, J. H. (2014). Crude Glycerol as Feedstock for the Sustainable Production of p-hydroxybenzoate by *Pseudomonas putida* S12. *New Biotechnology*, 31(1), 114–9.
- Vlassis, T., Antonopoulou, G., and Lyberatos, G. (2012). Anaerobic Treatment of Glycerin for Methane and Hydrogen Production. *Global NEST*, 14(2), 149–156.
- Vlysidis, A., Binns, M., Webb, C., and Theodoropoulos, C. (2011). Glycerol Utilisation for the Production of Chemicals: Conversion to Succinic Acid, a Combined Experimental and Computational Study. *Biochemical Engineering Journal*, 58-59, 1–11.
- Wahab, A. G. (2014). *Malaysia Biofuels Annual*. USDA Foreign Agricultural Service, *GAIN Report*, 1–10. Retrieved from [http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Biofuels%20Annual\\_Kuala%20Lumpur\\_Malaysia\\_6-25-2014.pdf](http://gain.fas.usda.gov/Recent%20GAIN%20Publications/Biofuels%20Annual_Kuala%20Lumpur_Malaysia_6-25-2014.pdf) on 14<sup>th</sup> December 2015.
- Wan Omar, W. N. N., and Amin, N. A. S. (2011). Biodiesel Production from Waste Cooking Oil Over Alkaline Modified Zirconia Catalyst. *Fuel Processing Technology*, 92(12), 2397–2405.
- Wang, J. L., and Wan, W. (2009). Kinetic Models for Fermentative Hydrogen Production: A Review. *International Journal of Hydrogen Energy*, 34(8), 3313–3323.
- Wang, J., and Wan, W. (2011). Combined Effects of Temperature and pH on Biohydrogen Production by Anaerobic Digested Sludge. *Biomass and Bioenergy*, 35(9), 3896–3901.
- Wohlgemut, O. (2011). *Co-Digestion of Hog Manure with Glycerol to Boost Biogas and Methane Production*. University of Manitoba.
- Won, S. (2013). Anaerobic Fermentation for Biological Hydrogen Production in a Sequencing Batch Reactor, PhD Thesis, The University of British Columbia.
- Wu, K. J., Lin, Y. H., Lo, Y. C., Chen, C. Y., Chen, W. M., and Chang, J. S. (2011). Converting Glycerol into Hydrogen, Ethanol, and Diols with a *Klebsiella sp.* HE1 Strain via Anaerobic Fermentation. *Journal of the Taiwan Institute of Chemical Engineers*, 42(1), 20–25.
- Xiao, Y., Zhang, X., Zhu, M., and Tan, W. (2013). Effect of the Culture Media Optimization, pH and Temperature on the Biohydrogen Production and the

- Hydrogenase Activities by *Klebsiella pneumoniae* ECU-15. *Bioresource Technology*, 137, 9–17.
- Yaakob, Z., Mohammad, M., Alherbawi, M., Alam, Z., and Sopian, K. (2013). Overview of the Production of Biodiesel from Waste Cooking Oil. *Renewable and Sustainable Energy Reviews*, 18, 184–193.
- Yang, F., Hanna, M. a, and Sun, R. (2012). Value-added Uses for Crude Glycerol-A By-product of Biodiesel Production. *Biotechnology for Biofuels*, 5(1), 13.
- Yazdani, S. S., and Gonzalez, R. (2007). Anaerobic Fermentation of Glycerol: A Path to Economic Viability for the Biofuels Industry. *Current Opinion in Biotechnology*, 18(3), 213–219.
- Zaher, U., Bouvier, J., Steyer, J., and Vanrolleghem, P. a. (2004). Titrimetric Monitoring of Anaerobic Digestion: VFA, Alkalinities and More. *Proceedings of 10th World Congress on Anaerobic Digestion (AD10)*, August, 330–336.
- Zhang, C., Su, H., Baeyens, J., and Tan, T. (2014). Reviewing the Anaerobic Digestion of Food Waste for Biogas Production. *Renewable and Sustainable Energy Reviews*, 38, 383–392.
- Zhang, Y., Dubé, M. a., McLean, D. D., and Kates, M. (2003). Biodiesel Production from Waste Cooking Oil: 1. Process Design and Technological Assessment. *Bioresource Technology*, 89(1), 1–16.