BIOHYDROGEN PRODUCTION BY A MICROBIAL CONSORTIUM ISOLATED FROM LOCAL HOT SPRING

SITI HALIMAH HASMONI

UNIVERSITI TEKNOLOGI MALAYSIA

BIOHYDROGEN PRODUCTION BY A MICROBIAL CONSORTIUM ISOLATED FROM LOCAL HOT SPRING

SITI HALIMAH BINTI HASMONI

A thesis submitted in fulfilment of the
requirement for the award of degree of
Doctor of Philosophy (Bioscience)

Faculty of Science
Universiti Teknologi Malaysia

SEPTEMBER 2018

As I lay in bed, watching my son and my husband asleep, I can't believe that Allah swt has granted me a family of my own…. This is for both of them…my family

ACKNOWLEDGMENT

Alhamdulillah, I am thankful to Allah S.W.T., the Almighty, the most Merciful, for his blessings and guidance throughout my PhD journey. My upmost gratitude to my supervisors; Assoc Prof Dr. Shafinaz Shahir and Prof Dr. Zaharah Ibrahim for their intellectual supports and guidance throughout my PhD research. My sincere appreciation to all laboratory staff in FBME especially En Yusnizam, En Hairol, En Afiezy and Puan Suhaidah Suleiman for their kindness and assistance during my laboratory work. My heartfelt gratefulness is dedicated to my friends, Puan Amalina Ramli for showing me guidance, Fahmi Mubarak for helping a noob big sister out, to my listening ear Munirah Ramli, to my little sister in the lab Nur Sahslin Irwan Shah, to Syamimi Md Jamal for helping me with my DGGE, to finally my peer with the same age as mine Noradilin Abdullah whom always is a friend in need and last but not least to my 'kakak' Nurliyana Ahmad Zawawi, with lots of wisdom and useful advices, thank you all for all the help and wonderful advices. To all the friends and acquaintances I made throughout my PhD journey, thank you for your kindness and help in easing in my PhD journey. My warmest gratitude is dedicated to my family and my in laws for the du'as and the support. Finally, my greatest and warmest and loads of appreciation and gratitude for my ever loving husband, Naeim for understanding and supporting me and loving me. Your love to me makes me stronger everyday and you made me be the better version of myself. Not forgetting my son, Rafiqi, half of me, all of me, it seems that you grew up in parallel with my PhD studies. You have given me so much joy and I look forward everyday to spend time with you ever since you are born. You are my greatest gift of all, to have you in my PhD journey is the best thing a student mom could ever asked for. #sayangrafiqi

ABSTRACT

Biohydrogen production from microorganism is a form of renewable energy that could supplement the depletion of fossil fuels. In producing biohydrogen, microbial consortia are more feasible than pure cultures because of its operational ease and stability and it is more favourable energetically at elevated temperatures which enables thermophiles to reach higher biohydrogen production than mesophiles. The aim of this study was to isolate, enrich and screen microbial consortium from local hot spring for its potential in producing biohydrogen, to optimize the selected consortium for optimal biohydrogen production and to identify the microbial diversity community of the consortium. Sampling was conducted at Gadek, Cherana Putih, Gersik and Selayang hot spring and the samples were enriched in Mineral Salt Succinate medium. The enriched consortia were screened for biohydrogen production using Gas Chromatography-Thermal Conductivity Detector (GC-TCD) and the biohydrogen production of the selected consortium was optimized by one factor at a time (OFAT) method. The kinetic analysis of the growth and biohydrogen production of the consortium were analyzed using the modified Logistic growth equation and modified Gompertz equation respectively. The microbial diversity community of the consortia were observed using 16S rRNA polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). To determine the microbial population dynamics of the consortia, 16S rRNA clone library were constructed for the consortia before and after optimization and sequencing data were analyzed using Mothur. Microbial consortium from Gadek hot spring (GDC) yielded the highest biohydrogen production compared to other consortia. The optimized condition (15% (v/v) inoculum size, 50°C, pH 7, 2 g/L sodium pyruvate and 0.5 g/L tryptone) showed a maximal biomass growth of 0.563 g dry cell weight/L and apparent specific growth rate of 0.959 h^{-1}. Whilst the optimized hydrogen production potential was 86.2 mmol H_2/L culture with the maximal production rate of 4.117 mmol/L h^{-1}, biohydrogen yield obtained was 135.7 mmol H_2/g biomass and the lag phase time was 5.1 hours. DGGE showed a slight microbial shift between the consortia before and after optimization. From the 16S rRNA clone library, 21 clones were obtained and a total of four operational taxonomic unit (OTU) were detected. Both consortia showed Firmicutes and Proteobacteria as the predominant phyla which have phylogeny affiliations to hydrogen producers. However, OTU_4 (*Sporoacetegenium mesophilum*) was only present in the consortium before optimization, OTU_1 (*Thauera* sp), OTU_2 (*Paenibacillus barengoltzii*) and OTU_3 (Sporomusaceae g. sp) were present in both consortia. Analysis showed the presence of OTU_2 and OTU_3 and the abundance of OTU_1 in the optimized consortium led to an increased in biohydrogen production of about 8 fold more from the consortium before optimization. In conclusion, this is the first study that reports a unique combination of *Thauera* sp., *Paenibacillus barengoltzii* and Sporomusaceae g. sp. which are able to produce a high amount of biohydrogen at the optimized condition.

ABSTRAK

Penghasilan biohidrogen daripada mikroorganisma ialah sejenis tenaga diperbaharui yang dapat menambah kekurangan sumber bahan api. Dalam menghasilkan biohidrogen, konsortia mikrob adalah lebih sesuai berbanding kultur tulen kerana operasi yang mudah dan kestabilannya dan ia lebih sesuai digunakan dari segi tenaga pada suhu tinggi yang membolehkan termofil mencapai penghasilan yang lebih tinggi berbanding mesofil. Tujuan kajian ini adalah untuk memencil, memperkaya, dan menyaring konsortium mikrob dari kolam air panas tempatan untuk keupayaanya menghasilkan biohidrogen. Selain itu, tujuan kajian ini juga adalah untuk mengoptimumkan konsortium terpilih bagi penghasilan biohidrogen yang optimum. dan untuk mengenal pasti kepelbagaian komuniti mikrob di dalam konsortium tersebut. Pensampelan dilakukan di kolam air panas Gadek, Cherana Putih, Gersik dan Selayang dan sampel diperkaya dalam medium garam mineral suksinat. Konsortia diperkayakan disaring untuk penghasilan biohidrogen dengan menggunakan Kromatografi Gas- Pengesan Terma Kekonduksian (GC-TCD) dan penghasilan biohidrogen oleh konsortium terpilih dioptimumkan dengan kaedah satu faktor pada satu masa (OFAT). Analisis kinetik terhadap pertumbuhan dan penghasilan biohidrogen oleh konsortium masing-masing dianalisis dengan menggunakan persamaan pertumbuhan Logistik terubah suai dan persamaan Gompertz terubah suai. Kepelbagaian komuniti mikrob dicerap dengan menggunakan tindak balas rantai polimerase16S rRNA-gel elektroforesis penyahaslian cerun (PCR-DGGE). Untuk menentukan populasi dinamik mikrob konsortia tersebut, perpustakaan klon 16S rRNA dibina untuk konsortia sebelum dan selepas pengoptimuman dan data penjujukan dianalisis dengan menggunakan Mothur. Konsortium mikrob dari kolam air panas Gadek (GDC) menghasilkan biohidrogen paling tinggi berbanding konsortia lain. Keadaan optimum (15% (v/v) saiz inokulum, 50°C, pH 7, 2 g/L natrium piruvat dan 0.5 g/L tripton) menunjukkan pertumbuhan biojisim maksimum sebanyak 0.563 g berat kering sel/L dan kadar pertumbuhan spesifik ketara, 0.959 h^{-1}. Sementara itu, penghasilan biohidrogen optimum adalah sebanyak 86.2 mmol H_2/L dengan kadar penghasilan maksimum 4.117 mmol/L h^{-1}, hasil biohidrogen adalah sebanyak 135.7 mmol H_2/g biojisim dan fasa lamban selama 5.1 jam. DGGE menunjukkan sedikit anjakan mikrob antara konsortia sebelum dan selepas pengoptimuman. Daripada perpustakaan klon 16S rRNA, 21 klon diperoleh dan sebanyak empat unit taksonomi operasi (OTU) dikesan. Kedua-dua konsortia menunjukkan Firmikut dan Proteobakteria sebagai filum pradominan yang mempunyai hubungan filogeni dengan penghasil hidrogen. Walau bagaimanapun, OTU_4 (*Sporoacetegenium mesophilum*) hanya terdapat pada konsortium sebelum pengoptimuman, OTU_1 (*Thauera* sp), OTU_2 (*Paenibacillus barengoltzii*) dan OTU_3 (Sporomusaceae g. sp) hadir dalam kedua-dua konsortia. Analisis menunjukkan kehadiran OTU_2 dan OTU_3 dan kelimpahan OTU_1 dalam konsortium yang dioptimumkan membawa kepada penghasilan biohidrogen meningkat lebih kurang 8 kali ganda daripada konsortium sebelum pengoptimuman. Kesimpulannya, ini adalah kajian pertama yang melaporkan kombinasi unik *Thauera* sp., *Paenibacillus barengoltzii* dan Sporomusaceae g. sp. yang mampu menghasilkan biohidrogen dengan jumlah yang tinggi pada keadaan optimum.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP	-	Adenosine triphosphate
BLAST	-	Basic Local Alignment Search Tool
DGGE	-	Denaturing Gradient Gel electrophoresis
DNA	-	Deoxyribonucleic acid
GC-TCD	-	Gas Chromatography Thermal Conductivity Detector
LH	-	light harvesting
NADH	-	Nicotinamide adenosine dinucleotide
OD	-	optical density
OFAT	-	one factor at a time
PCR	-	polymerase chain reaction
PNSB	-	Purple non sulphur bacteria
RC	-	reaction centre
RNA	-	Ribonucleic Acid
rRNA	-	ribosomal ribonucleic acid
SCE	-	Substrate conversion efficiency
TAE	-	Tris- acetate-EDTA
TCA	-	tricarboxylic acid
UV	-	ultraviolet

LIST OF SYMBOLS

% (v/v)	-	percentage volume per volume
°C	-	celcius
bp	-	base pair
h	-	hour
g	-	gram
g/L	-	gram per litre
kb	-	kilo base pair
kPa	-	kilo Pascal
L	-	litres
µL	-	microliter
mL	-	mililiter
min	-	minutes
mM	-	milimolar
ng/L	-	nanogram per litre
nm	-	nanometer
rpm	-	rotation per minute
Tm	-	melting point
V	-	volt

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Currently, fossil fuels such as coal, oil and natural gas are massively used for industrialization, transportations, generation of electricity, and overall the sole global energy (Huntley and Redalje, 2007; Hallenback *et al.*, 2009; Chandrasekhar *et al*., 2015). However, enormous consumption of fossil fuel has caused major environmental destruction, changes in global climate, global warming, emission of greenhouse gasses and health problems (Chang *et al*., 2006; Jamali *et al*., 2016). Hydrogen is one of the most abundant elements in the universe in its ionic form and is odourless, colourless, tasteless and non-poisonous gas (Das *et al*. 2001; Chong *et al*., 2009). Thus, it is recommended that hydrogen could replace fossil fuels and minimize the environmental pollution because of its clean and renewable energy properties.

Hydrogen gas is the simplest element and is the most abundance element in the universe. The atmosphere contains 0.07 % of hydrogen and the Earth's surface has 0.14 % of hydrogen (Das *et al*., 2001). Furthermore, hydrogen is a promising energy carrier of the future and can be derived from a variety of energy sources. Hydrogen is used in fuel cells with high efficiency of 142.35 kJ/g which means that on burning 1 g of hydrogen, 142.35 kJ of energy is produced (Singh, 2013). Hydrogen can either be

used as the fuel for direct combustion of engine or as the fuel for a fuel cell (Das and Veziroglu, 2008). Moreover, hydrogen is categorized as a clean fuel because upon oxidation, it only produces water which can be recycled again to produce more hydrogen, thus making it a source of renewable energy (Singh and Wahid, 2015). Furthermore, by only producing water upon combustion or oxidation hydrogen is a non-polluting and carbon-free alternative in comparison to fossil fuels which produces carbon dioxide upon combustion thus further increase the effects of greenhouse gases (Singh, 2013; Singh and Wahid, 2015).

Currently, hydrogen is used for hydrogenation of many products, foods, and ammonia for fertilizer and is also used in the petroleum industries (Das and Veziroglu, 2008; Kim and Kim, 2012). Furthermore, it has been reported that the demand for hydrogen is increasing and hydrogen could be the future of energy for power and transportation due to its many advantageous trait (Singh, 2013). Thus, a significant use of hydrogen has been demonstrated in the recent years for hydrogen-fueled transit buses, ships and submarines, including chemical and petrochemical applications (Singh and Wahid, 2015). However, unlike fossil fuels, hydrogen gas is not readily available in nature, and the commonly used production methods are quite expensive (Singh and Wahid, 2015). At present 40% hydrogen is produced from natural gases, 30% from heavy oil and naphtha, 18% from coal, 4% from electrolysis and about 1% from biomass (Sinha and Pandey, 2011). Most of this hydrogen production are exclusively made by methane steam reforming and coal gasification by using fossil fuels, which emits a significant amount of greenhouse gases (Kim and Kim, 2012). Therefore, renewable energy sources have to be employed for sustainable hydrogen production. Thus, biological hydrogen production are becoming important due to its renewable energy resources and its ability to operate at ambient temperature and atmospheric pressure (Wang and Wan, 2009; Loss *et al*., 2013).

Biohydrogen production are mainly from microorganisms that are able to produce hydrogen inside its metabolic pathway such as the photosynthetic bacteria (purple non sulfur bacteria (PNSB), cyanobacteria, purple sulfur bacteria) and fermentative hydrogen production bacteria (*Clostridium* sp). Both of these type of bacteria are extensively isolated and studied in mesophilic condition for its ability to produce high amount of hydrogen. However, Lazaro *et al.* (2015) concluded that mix cultures are more feasible in producing hydrogen in comparison to the pure cultures.

Furthermore, they also observed that mixed cultures are the preferred choice because of its operational ease, stability, diversity of biochemical functions, and the possibility to use a wide range of substrates (Han *et al*., 2012). Apart from that, it is known that biological hydrogen production is more favourable energetically at elevated temperatures which enables thermophiles to reach higher hydrogen production than mesophiles (Pawar and van Niel, 2013).

Hot Springs in Malaysia is a known hotspot for tourism. According to Baioumy *et al*. (2015), there are more than sixty hot springs in West Malaysia with the variation temperature of 41°C to 99°C and pH values varies in the range of 4.5 to 9.9 and the hot springs in West Malaysia are non-volcanic hot springs. However, biological studies are rare because of the lack comprehensive information of their microbial communities. Thus far, Chan *et al.* (2017) via independent cultivation, reported that generally, Firmicutes and Proteobacteria dominated the bacterial communities in all hot springs along the flank of the Banjaran Titiwangsa mountain range. For the past 5 years, a few of bacterial species has been successfully isolated from Malaysian hot spring which are *Rhodomicrobium vannielii* (Ainon *et al*., 2006), *Geobacillus thermoleovorans* CCB_US3_UF5 (Sakaff *et al*., 2012), and Sulphur oxidizing bacteria isolate (Hidayat *et al*., 2017). Furthermore, hot springs in Thailand (Puhakka *et al.*, 2012) and Turkey (Jessen *et al*., 2015) observe the existence of hydrogen producer bacteria which produces high yield of hydrogen. Nevertheless, to the best of our knowledge, there are no reports in the public domain regarding the ability of bacteria from hot springs in Malaysia to produce hydrogen. However, Ainon *et al*., (2006) reported the presence of a PNSB, *Rhodomicrobium vannielii*, a PNSB from Gadek hot spring in Melaka. PNSB is known to possess a metabolic pathway to produce hydrogen. Therefore in the present study, microbes were isolated from a few hot springs in Malaysia to explore their ability to produce biohydrogen. Furthermore, it would be beneficial to produce hydrogen in a higher scale for a future of clean and renewable energy source.

1.2 Problem Statement

Energy is the most vital source and is used daily for electricity, transportation, technology, manufacturing and industrialization. To date, these global energy requirements are heavily dependent on fossil fuels such as oil, coal and natural gaseous. There is an urgency to search for replacement source of energy since the depletion of limited fossil fuels source is inevitable. Furthermore, the global warming and the climate change that the world is enduring right now is causing worry due to the extensive use of fossil fuels where there is a tremendous emission of carbon dioxide during combustion of fossil fuels (Chong *et al*., 2009). Therefore, for these reasons, researches are looking for alternative fuels that could tackle the environmental issues mentioned. Thus, hydrogen is the best substitute for fossil fuel due to its abundance in the environment and it is a form of renewable energy (Jamali *et al*., 2016). This is because production of hydrogen only produces water upon oxidation and the water could be recycled again to produce hydrogen (Singh and Wahid, 2015). Hence, making it a form of renewable energy. Also it is a clean energy source due to the lack of emission of the greenhouse gases in the process of producing hydrogen and thus making it environmentally friendly (Singh, 2013; Singh and Wahid, 2015; Jamali *et al*., 2016). Moreover, biological process in producing hydrogen is an eco-friendly method which uses microorganisms via biochemical pathway in comparison to the conventional method which uses about 98% of fossil fuels (Singh and Wahid, 2015).

However, there are some limitations in production of biohydrogen such as:

i. Limitations of biohydrogen production in pure cultures

In recent years, studies on biohydrogen production via pure cultures are more leaning towards modifications of its genetic information and its metabolic pathway (Cai and Wang, 2014; Mohd Yasin *et al*., 2013; Ma *et al*., 2012; Rey *et al*, 2007; Morimoto *et al*., 2005; Kondo *et al*., 2002) .Thus, making the hydrogen production costly and prevents the commercial application of the technology (Cai *et al*., 2012). Furthermore, pure cultures require sterile conditions and strict control of environmental conditions making it difficult for large-scale process in producing hydrogen for future energy source (de Sá *et al*., 2013). In comparison, mix cultures are easy to control, due to the

absence of of sterilization and being adaptive to variations in feedstock or condition due to its interaction between different microorganisms in the mix culture making it favourable for large-scale processing (Bao *et al*., 2012; Loss *et al*., 2013; Sivagurunathan *et al*., 2014; Zhang *et al*., 2015). In addition, mix cultures are robust and able to convert a wide array of substrates because of their metabolic flexibility to utilize short-chain fatty acids and carbon dioxide and produce hydrogen (Shanmugam *et al.*, 2014). Thus, resulting in mix cultures and co-cultures producing higher hydrogen production rather than pure cultures without any genetic modifications (Zhang *et al.*, 2015).

ii. Abundance of different types of bacteria in a mix culture could lead to instability of biohydrogen production system.

To date, biohydrogen producing enriched consortia are mainly isolated from Palm Oil Mill Effluent (POME) which produces hydrogen in dark fermentation (Jamali and Md Jahim, 2016; Rasdi *et al*., 2009; Singh *et al*., 2013; Vijayaraghavan and Ahmad, 2006; Yossan *et al*., 2012). However, there is an underlying problem whereby instability of the consortium isolated from POME occurs due to the abundance of bacterial community (Singh and Wahid, 2015). The abundance of bacterial community usually consist of hydrogen consumers such as methanogens, homoacetogens, sulphate and nitrate reducing bacteria (Singh, 2013). Furthermore, this resulted in depleting the amount of hydrogen yield from 11% to 43% due to these bacteria consuming hydrogen in the mix culture (Saady, 2013). In addition, low hydrogen yield were reported due to the less efficiency in converting substrates to hydrogen because most thermal enthalpies are lost in the formation of volatile fatty acids (VFA) (Wong *et al*., 2005). Thus, another environmental source are needed for the production of hydrogen with an optimal bacterial community to produce high yield of hydrogen.

iii. Lack of data for local thermophilic isolates and their biohydrogen production

Hydrogen production by mix culture has shown to be higher at higher temperatures (O-Thong *et al*., 2008; Akutsu *et al.*, 2009; Puhakka *et al*., 2012; Zhang *et al*., 2016). Thus, it is possible that thermophilic enrich culture may be capable of producing high

amount of hydrogen. Enrich cultures taken from the hot springs reportedly yield high amount of hydrogen (Prasertsan and O-Thong, 2011; Puhakka *et al*., 2012; Phummala *et al*., 2014). At thermophilic condition, the hydrogen community producer becomes energetically favorable and hydrogen consuming reactions become less favorable, better pathogenic destruction, higher rate of hydrolysis and thus higher hydrogen yields (Das, 2001; Chong *et al*., 2009; Md Jahim, 2016; Roy *et al*., 2014). However, although the thermophiles are cultivated at elevated temperatures with highly intensive energy requirements, their hydrogen production can be closer to the theoretical yield in comparison to mesophiles by overwhelming the thermodynamic barrier (Chandrasekhar *et al*., 2015). Thus, a higher temperature is more feasible for hydrogen production due to favorable thermodynamics and hold tremendous promise for the forthcoming generations as well as for the commercial production of hydrogen fuel (Hasyim *et al*., 2011). However, to date, in the public domain, research done to investigate the ability of the microbial community of the hot spring in producing hydrogen are sparse.

iv. Lack of data in bacterial consortium containing PNSB for biohydrogen production via photo fermentation

As for photo fermentation in biohydrogen production, it usually involves PNSB that utilizes sunlight to oxidize organic compounds and generate the electron potential needed to drive hydrogen production (Azwar *et al*., 2014). Additionally, photo fermentation is widely used for wastewater remediation and stabilization due to its versatility in sources of metabolic substrate (Ghadamshetty *et al*., 2008; Kim and Kim, 2012). Thus, solar energy can be utilized in producing hydrogen with minimal non-renewable energy inputs and by utilizing low cost substrates or waste streams and, by collecting and recycling useful by-products (Gadhamshetty *et al*., 2008). Also, PNSB can potentially divert 100% of electrons from an organic substrate to hydrogen production (Azwar *et al*., 2014). Hence, biohydrogen production via photo fermentation is favourable due to its potential in producing high amount of hydrogen and its versatility in consuming various substrate which could be utilized as wastewater remediation apart from producing hydrogen. Thus far, consortium containing PNSB for one step biohydrogen production has been reported from only the mesophilic environment (Yanling *et al*., 2008, Loss *et al*., 2013 and Lazaro *et al*., 2015). Also, there are no studies yet available in the public domain using consortium containing PNSB isolated from the hot springs for biohydrogen production.

Therefore, in this research, microbes from hot springs samples were isolated and further analyzed its biohydrogen production potential and its environmental effect on biohydrogen production. In addition, the microbial diversity of the mix culture taken from the hot spring is further studied to understand its role and its abundance in a consortium. Overall, in this present study, consortium from hot spring were assessed for their ability to produce hydrogen for future energy source.

1.3 Research Objectives

This study was carried out to investigate the ability of an enriched mix bacterial culture (consortium) from a local hot spring to produce high amount of biohydrogen and identify the microbial population that is responsible for hydrogen production. The specific objectives of this studies were:

1) To isolate, enrich, characterize and screen the consortium for biohydrogen production from various hot springs samples.
2) To characterize and optimize the maximal biohydrogen production of the selected consortium in batch mode.
3) To identify the microbial community diversity of the consortium before and after biohydrogen production optimization.

1.4 Scope of Study

In this study, water samples from Selayang hot spring, Selangor, Cherana Putih and Gadek hot springs in Melaka were enriched in a medium for PNSB with the aim of obtaining a consortium with PNSB. This is because PNSB are versatile and able to break down any organic substrate into hydrogen production whilst having the potential to divert 100% of electrons from an organic substrate to hydrogen production (Azwar *et al*., 2014). Apart from producing hydrogen, PNSB could simultaneously remediate

wastewater due to its ability in utilizing any form of substrate and also its ability to survive in an extreme habitat (Seifert *et al*., 2010; Seifert and Zagrodnik, 2009). Chan *et al*. (2017) reported that the dominant phyla in most of Malaysian hot springs are Proteobacteria. PNSB is from the phyla of Proteobacteria. Hence, it could be hypothesize that PNSB from hot springs could produce a high amount of hydrogen due to its efficiency in converting its substrate to hydrogen (Azwar *et al*., 2014). Moreover, at elevated temperatures, production can be closer to the theoretical yield by overwhelming the thermodynamic barrier (Chandrasekhar *et al*., 2015).

Next, the various consortium were screened for its ability to produce hydrogen via gas chromatography-thermal conductivity detector (GC-TCD). Isolation and identification of the consortium with the highest hydrogen production was done and the isolated pure cultures were screened for hydrogen production. The environmental effects on hydrogen production of the consortium was further studied. Parameters such as the light illumination protocol, inoculum size, initial pH of the medium, incubation temperature, effects of carbon sources and its concentration and effects of nitrogen sources and its concentration which influence the hydrogen production of the consortium were optimized conventionally using one factor at a time method (OFAT). The biohydrogen production of the consortium was kinetically analyzed using the modified Gompertz equation and the growth and biomass production of the consortium was analyzed using the Logistic growth model. Furthermore, the relationship between biomass growth and the production of hydrogen by the consortium were also analyzed. The microbial community diversity of the consortium before and after optimization of hydrogen production was done via denaturing gradient gel electrophoresis to screen a microbial shift in bacterial population. Then, identification of the microbial population of the consortium before and after optimization of hydrogen production was done by construction of 16S rRNA gene clone library and analyzed using Bioedit and Mothur.

1.5 Significance of Study

This study provides information of the ability of a bacterial consortium taken from the hot spring to produce hydrogen. Biohydrogen production has been vastly studied around the world, however new information arises that thermophilic bacterial consortium produce much higher hydrogen production (Shin *et al*., 2004; Puhakka *et al*., 2012; Zhang *et al*., 2016). Only a few studies were found in the public domain on the ability of bacterial communities taken from hot springs to produce hydrogen such as Puhakka *et al.* (2012) and Jessen *et al*. (2015). Thus, in this research, biohydrogen production from bacterial culture of hot springs in Malaysia has been studied to further provide the information of the ability of a bacterial consortium in hot spring Malaysia to produce hydrogen.

This research will also provide important environmental parameters and its kinetic analysis in enhancing the production of hydrogen in the consortium. The production of hydrogen is influenced by many factors such as temperature, pH, its carbon source and also its nitrogen source. Different type of microorganisms and consortiums has different effects on hydrogen production and its growth base on the variation of the environmental parameters. Furthermore, kinetic models were developed and applied for growth and hydrogen production to describe the progress of growth and hydrogen production process respectively. It is unknown whether a certain combination of many environmental parameters could yield high amount of hydrogen by the bacterial consortium of the hot spring. Thus, by investigating the environmental factors and its kinetic analysis, it will provide a better evaluation of the kinetic growth and hydrogen production of the consortium and its effects towards different environmental parameters.

Furthermore, this study provides an in depth analysis of the microbial community dynamics of the consortium that is responsible for producing high hydrogen production. Knowledge of the microbial composition of the major hydrogen producing microorganisms would result in efficient and optimal operation of fermentative hydrogen producing systems (O-Thong *et al*., 2008). Apart from identifying the bacterial consortium, little information concerning microbial population structures and its dynamic changes in hydrogen production is available. Therefore in this research,

REFERENCES

Abdeshahian, P., Al-Shorgani, N. K. N., Salih, N. K. M., Shukor, H., Kadier, A., Hamid, A. A., and Kalil, M. S. (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.

Abo-Hashesh, M., and Hallenbeck, P. C. (2012). Microaerobic dark fermentative Hydrogen production by the photosynthetic bacterium, *Rhodobacter capsulatus* JP91. International *Journal of Low-Carbon Technologies*, 7(2), 97 103.

Ainon, H. , Tan, C. J. and Vikineswary, S. (2006). Biological Characterization of *Rhodomicrobium vannielii* Isolated from a Hot Spring at Gadek , Malacca , Malaysia. *Water*, 2(1), 15–21.

Akroum-Amrouche, D., Abdi, N., Lounici, H., and Mameri, N. (2011). Effect of physico-chemical parameters on biohydrogen production and growth characteristics by batch culture of *Rhodobacter sphaeroides* CIP 60.6. *Applied Energy*, 88(6), 2130–2135.

Akutsu, Y., Li, Y., Harada, H., and Yu., H. (2009), Effects of temperature and substrate concentration on biological hydrogen production from starch. *International Journal of Hydrogen Energy*, 34(6), 2558-2566.

Alvarado-Cuevas, Z. D., Acevedo, L. G. O., Salas, J. T. O., and De León-Rodríguez, A. (2013). Nitrogen sources impact hydrogen production by Escherichia coli using cheese whey as substrate. *Biotechnology for the Bio and Green Economy*, 30(6), 585–590.

An, D., Li, Q., Wang, X., Yang, H., and Guo, L. (2014). Characterization on hydrogen production performance of a newly isolated *Clostridium beijerinckii* YA001 using xylose. *International Journal of Hydrogen Energy*, 39(35), 19928-19936.

Anon, (2005), DGGE HELP: A DGGE Guide. Retrieved from http://ddgehelp.blogspot.ca/2005/11/dgge-guide.html

Anzola-Rojas, P., Gonçalves, S., Canedo, C., Maia, V., Oliveira, D., and Zaiat, M. (2015). The use of the carbon / nitrogen ratio and specific organic

loading rate as tools for improving biohydrogen production in fixed-bed reactors. *Biotechnology Reports*, 5, 46–54.

Ave, N. M. (2002). Hydrogen Production by Anaerobic Microbial Communities Exposed to Repeated Heat Treatments. *Proceedings of the 2002 U.S. DOE Hydrogen Program Review NREL/CP-610-32405*, 1–17.

Azman, N. F., Abdeshahian, P., Kadier, A., Nasser Al-Shorgani, N. K., Salih, N. K. M., Lananan, I., and Kalil, M. S. (2016). Biohydrogen production from de-oiled rice bran as sustainable feedstock in fermentative process. *International Journal of Hydrogen Energy*, 41(1), 145–152

Azwar, M. Y., Hussain, M. A., and Abdul-Wahab, A. K. (2014). Development of biohydrogen production by photobiological, fermentation and electrochemical processes: *A review. Renewable and Sustainable Energy Reviews*, 31, 158-173.

Bahari, Z. B. M. (2016). Characterization of Arsenate Reduction by Arsenic Tolerant *Microbcterium foliorum* Strain SZ1 Isolated from Gold Ores.PhD Thesis. Universiti Teknologi Malaysia, Skudai.

Bao, M., Su, H., and Tan, T. (2012). Biohydrogen Production by Dark Fermentation of Starch Using Mixed Bacterial Cultures of *Bacillus* sp and *Brevumdimonas* sp. *Energy & Fuels*, 26(9), 5872–5878.

Bao, M. D., Su, H. J., and Tan, T. W. (2013). Dark fermentative bio-hydrogen production: Effects of substrate pre-treatment and addition of metal ions or L-cysteine. *Fuel*, 112, 38–44.

Baranyi, J. (2010). Modelling and parameter estimation of bacterial growth with distributed lag time PhD Thesis. University of Szeged , Hungary.

Barton, L., and Northup, D. (2011). Microbial Ecology: Chapter 5: The how of microbial ecology studies. *In Microbial Ecology* .131–158

Basak, N., and Das, D. (2007). The prospect of purple non-sulfur (PNS) photosynthetic bacteria for hydrogen production: The present state of the art. *World Journal of Microbiology and Biotechnology*, 23, 31–42.

Buchanan, R. ., Whiting, R. ., and Damert, W. (1997). When is simple good enough: a comparison of the Gompertz, Baranyi, and three-phase linear models for fitting bacterial growth curves. *Food Microbiology*, 14(4), 313–326.

Bundhoo, M.A.Z. and Mohee, R., 2016. Inhibition of dark fermentative bio-hydrogen production: A review. *International Journal of Hydrogen Energy*, 41(16), 6713–

6733.

Cai, J., and Wang, G. (2012). Hydrogen production by a marine photosynthetic bacterium, *Rhodovulum sulfidophilum* P5, isolated from a shrimp pond. *International Journal of Hydrogen Energy*, 37(20), 15070–15080.

Cai, J., Wang, G., and Pan, G. (2012). Hydrogen production from butyrate by a marine mixed phototrophic bacterial consort. *International Journal of Hydrogen Energy*, 37(5), 4057–4067.

Cai, J., and Wang, G. (2014). Photo-biological hydrogen production by an acid tolerant mutant of *Rhodovulum sulfidophilum* P5 generated by transposon mutagenesis. *Bioresource Technology*, 154, 254–259.

Castillo, P., Magnin, J. P., Velasquez, M., and Willison, J. (2012). Modeling and optimization of hydrogen production by the photosynthetic bacterium *Rhodobacter capsulatus* by the methodology of Design of Experiments (DOE): Interaction between lactate concentration and light luminosity. *Energy Procedia*, 29, 357–366.

Chan, C.S., Chan, G. K., Tay, Y-L., Chua, Y-H and Goh, K. M. (2015). Diversity of thermophiles in a Malaysian hot spring determined using 16S rRNA and shotgun metagenome sequencing. *Frontiers in Microbiology*, 6(MAR), pp.1-15

Chan, C. S., Chan, K. G., Ee, R., Hong, K. W., Urbieta, M. S., Donati, E. R.,and Goh, K. M. (2017). Effects of physiochemical factors on prokaryotic Biodiversity in Malaysian circumneutral hot springs. *Frontiers in Microbiology*, 8.

Chandrasekhar, K., Lee, Y., and Lee, D. (2015). Biohydrogen Production : Strategies to Improve Process Efficiency through Microbial Routes. *International Journal of Molecular Sciences*, 8266–8293.

Chang, J. J., Chen, W. E., Shih, S. Y., Yu, S. J., Lay, J. J., Wen, F. S., and Huang, C. C. (2006). Molecular detection of the clostridia in an anaerobic biohydrogen fermentation system by hydrogenase mRNA-targeted reverse transcription-PCR. *Applied Microbiology and Biotechnology*, 70(5), 598–604.

Chen, S., Song, L., and Dong, X. (2006). *Sporacetigenium mesophilum* gen. nov., sp. nov., isolated from an anaerobic digester treating municipal solid waste and sewage. *International Journal of Systematic and Evolutionary Microbiology*, 56(4), 721–725.

Chen, W. (2006). Biological hydrogen production by anaerobic fermentation. PhD Thesis. Iowa State University, Ames, Iowa

Cheng, C.-H., Hung, C.-H., Lee, K.-S., Liau, P.-Y., Liang, C.-M., Yang, L.-H., and Lin, C.-Y. (2008). Microbial community structure of a starch-feeding fermentative hydrogen production reactor operated under different incubation conditions. *International Journal of Hydrogen Energy*, 33(19), 5242–5249.

Cheng, J., Su, H., Zhou, J., Song, W., and Cen, K. (2011). Hydrogen production by mixed bacteria through dark and photo fermentation. *International Journal of Hydrogen Energy*, 36(1), 450–457.

Cho, Y., and Lee, T. (2011). Variations of hydrogen production and microbial community with heavy metals during fermentative hydrogen production. *Journal of Industrial and Engineering Chemistry*, 17(2), 340–345.

Chong, M. L., Sabaratnam, V., Shirai, Y., and Hassan, M. A. (2009). Biohydrogen production from biomass and industrial wastes by dark fermentation. *International Journal of Hydrogen Energy*, 34(8), 3277–3287.

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

Chow, W.S., Irawan, S. and Fathaddin, M.T., (2010). Hot Springs in the Malay Peninsula. In *Proceedings World Geothermal Congress 2010*. pp. 25–29.

Ciranna, A., Santala, V., and Karp, M. (2011). Biohydrogen production in alkalithermophilic conditions: *Thermobrachium celere* as a case study. Bioresource Technology, 102(18), 8714–8722.

Cole, J.R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., Brown, C. T., Porras-Alfaro, A., Kuske, C. R., Tiedje, J. M. (2014). Ribosomal Database Project: Data and tools for high throughput rRNA analysis. *Nucleic Acids Research*, 42(D1), 633–642.

Cottrell, M. T., and Kirchman, D. L. (2009). Photoheterotrophic microbes in the arctic ocean in summer and winter. *Applied and Environmental Microbiology*, 75(15), 4958–4966.

D'Haene, S. E., Crouch, L. I., Jones, M. R., and Frese, R. N. (2014). Organization in photosynthetic membranes of purple bacteria in vivo: The role of carotenoids. *Biochimica et Biophysica Acta - Bioenergetics*, 1837(10), 1665–1673.

d'Ippolito, G., Dipasquale, L., Vella, F. M., Romano, I., Gambacorta, A., Cutignano,

A., and Fontana, A. (2010). Hydrogen metabolism in the extreme thermophile *Thermotoga neapolitana. International Journal of Hydrogen Energy*, 35(6), 2290–2295.

Dan, D., Zhang, D. Peng, Liu, W. Cheng, Lu, C-g, and Zhang, T-T. (2014). Diversity Analysis of Bacterial Community from Permafrost Soil of Mo-he in China. *Indian Journal of Microbiology*, 54(1), 111–113.

Das, D., Nejat, T., and Glu, V. (2001). Hydrogen production by biological processes: a survey of literature. *International Journal of Hydrogen Energy*, 26, 13–28.

Das, D., and Veziroglu, T. (2008). Advances in biological hydrogen production processes. *International Journal of Hydrogen Energy*, 33(21), 6046–6057.

Das, D. (2009). Advances in biohydrogen production processes: An approach towards commercialization. *International Journal of Hydrogen Energy*, 34(17), 7349-7357.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber,T., Dalevi, D. ,Hu, P. andAndersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, 72(7),5069–5072.

de Hollander, J.A., (1993). Kinetics of microbial product formation and its consequences for the optimization of fermentation processes. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, 63(3–4),.375–381.

de Sá, L. R. V., Cammarota, M. C., de Oliveira, T. C., Oliveira, E. M. M., Matos, A., and Ferreira-Leitão, V. S. (2013). Pentoses, hexoses and glycerin as substrates for biohydrogen production: An approach for Brazilian biofuel integration. *International Journal of Hydrogen Energy*, 38(7), 2986–2997.

Disnard, J., Beaulieu, C., and Villemur, R. (2011). Composition of the bacterial biota in slime developed in two machines at a Canadian paper mill. Canadian Journal of Microbiology, 57(2), 91–104.

Dolly, S., Pandey, A., Pandey, B. K., & Gopal, R. (2015). Process parameter optimization and enhancement of photo-biohydrogen production by mixed culture of *Rhodobacter sphaeroides* NMBL-02 and Escherichia coli NMBL-04 using Fe-nanoparticle. *International Journal of Hydrogen Energy*, 40(46), 16010–16020.

Drews, G. (1996). Forty-five years of developmental biology of photosynthetic bacteria. *Photosynthesis Research*, 48, 325–352.

Elsharnouby, O., Hafez, H., Nakhla, G., and El Naggar, M. H. (2013). A critical literature review on biohydrogen production by pure cultures. *International Journal of Hydrogen Energy*, 38(12), 4945–4966.

Etchebehere, C., Castelló,E., Wenzel,J., Anzola-Rojas, M. d. P., Borzacconi, L., Buitrón, G., Cabrol, L., Carminato, V. M., Carrillo-Reyes, J., Cisneros-Pérez , C., Fuentes, L., Moreno-Andrade , I., Razo, E., and Z. M. (2016). Microbial communities from 20 different hydrogen-producing reactors studied by 454 pyrosequencing. *Applied Microbiology and Biotechnology*, 100(7), 3371-3384.

Energy Information Administration. U.S Department of Energy, (2008). *The Impact of Increased Use of Hydrogen on Petroleum Consumption and Carbon Dioxide Emissions*.

Fang, H. H. P., and Liu, H. (2002). Effect of pH on hydrogen production from glucose by a mixed culture. *Bioresource Technology*, 82(1), 87–93.

Fang, H. H. P., Zhang, T., and Li, C. (2006). Characterization of Fe-hydrogenase genes diversity and hydrogen-producing population in an acidophilic sludge. *Journal of Biotechnology*, 126(3), 357–364.

Fang, H., Liu, H., and Zhang, T. (2005). Phototrophic hydrogen production from acetate and butyrate in wastewater. *International Journal of Hydrogen Energy*, 30(7), 785–793.

Ferchichi, M., Crabbe, E., Hintz, W., Gil, G. H., and Almadidy, A. (2005). Influence of culture parameters on biological hydrogen production by *Clostridium saccharoperbutylacetonicum* ATCC 27021. *World Journal of Microbiology and Biotechnology*, 21(6–7), 855–862.

Ferris, M. J., and Hirsch, C. F. (1991). Method for isolation and purification of cyanobacteria. *Applied and Environmental Microbiology*, 57(5), 1448–1452.

Foss, S., and Harder, J. (1998). *Thauera linaloolentis* sp. nov. and *Thauera terpenica* sp. nov., Isolated on Oxygen-containing Monoterpenes (Linalool, Menthol, and Eucalyptol and Nitrate. *Systematic and Applied Microbiology*, 21(3), 365-373.

Furukawa, S., Suzuki, R., Ochi, K., Yashima, T., and Komatsu, T. (2015). Hydrogen Production from Aqueous Solutions of Urea with Ruthenium-based Catalysts. *ChemSusChem Communications*, 8(12), 2028–2030.

Gadhamshetty, V., Sukumaran, A., Nirmalakhandan, N., and Theinmyint, M. (2008). Photofermentation of malate for biohydrogen production— A modeling approach. *International Journal of Hydrogen Energy*, 33(9), 2138–2146.

Gadhe, A., Sonawane, S. S., and Varma, M. N. (2014). Kinetic analysis of biohydrogen production from complex dairy wastewater under optimized condition. *International Journal of Hydrogen Energy*, 39(3), 1306–1314.

Ghatak, M. Das, Mahanta, P., and Straw, R. (2014). Kinetic Assessment of Biogas Production from Lignocellulosic Biomasses. *International Journal of Engineering and Advanced Technology (IJEAT)*, 3(5), 244–249.

Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P. N. L., and Esposito, G. (2015). A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products. *Applied Energy*, 144, 73–95.

Gilbert, J. J., Ray, S., and Das, D. (2011). Hydrogen production using *Rhodobacter sphaeroides* (O.U. 001) in a flat panel rocking photobioreactor. *International Journal of Hydrogen Energy*, 36(5), 3434–3441.

Gomelsky, M., and Hoff, W. D. (2011). Light helps bacteria make important lifestyle decisions. *Trends in Microbiology*.19. 441-448

Gupta, R.S., 2003. Evolutionary relationships among photosynthetic bacteria. *Photosynthesis research*, 76(1–3), 173–83.

Hallenbeck, P. C. (2012.). Microbial Technologies in Advanced Biofuels Production. Springer.London.

Hallenbeck, P. C. (2013). Biohydrogen. Biohydrogen. Elsevier.USA.

Hallenbeck, P. C., and Liu, Y. (2015). Recent advances in hydrogen production by photosynthetic bacteria. *International Journal of Hydrogen Energy*, 41(7), 4446–4454.

Han, H., Liu, B., Yang, H., and Shen, J. (2012). Effect of carbon sources on the photobiological production of hydrogen using *Rhodobacter sphaeroides* RV. *International Journal of Hydrogen Energy*, 37(17), 12167–12174.

Han, W., Ye, M., Zhu, A. J., Huang, J. G., Zhao, H. T., and Li, Y. F. (2015). A combined bioprocess based on solid-state fermentation for dark fermentative

hydrogen production from food waste. *Journal of Cleaner Production*, 112, 3744–3749.

Hartman, A.L. Riddle, S., McPhillips, T., Ludäscher, B., Eisen, J. A.., (2010). Introducing W.A.T.E.R.S.: A Workflow for the Alignment, Taxonomy, and Ecology of Ribosomal Sequences. *BMC Bioinformatics*, 11.(317),1-14

Hasyim, R., Imai, T., O-Thong, S., and Sulistyowati, L. (2011). Biohydrogen production from sago starch in wastewater using an enriched thermophilic mixed culture from hot spring. *International Journal of Hydrogen Energy*, 36(21), 14162–14171.

He, D., Bultel, Y., Magnin, J.-P., and Willison, J. C. (2006). Kinetic analysis of photosynthetic growth and photohydrogen production of two strains of *Rhodobacter Capsulatus*. *Enzyme and Microbial Technology*, 38(1–2), 253–259.

Hidayat, M. Y., Saud, H. M., and Samsudin, A. A. (2017). Isolation and characterisation of Sulphur oxidizing bacteria isolated from hot spring in Malaysia for biological deodorisation of hydrogen sulphide in chicken Manure. *Media Peternakan*, 40(3).

Hitit, Z. Y., Lazaro, C. Z.,and Hallenbeck, P. C. (2017). Hydrogen production by co-cultures of *Clostridium butyricum* and *Rhodospeudomonas palustris*: Optimization of yield using response surface methodology. *International Journal of Hydrogen Energy*, 2(42). 6578 6589

Hniman, A., Prasertsan, P., and O-Thong, S. (2011). Community analysis of thermophilic hydrogen-producing consortia enriched from Thailand hot spring with mixed xylose and glucose. *International Journal of Hydrogen Energy*, 36(21), 14217–14226.

Hu, X., Ritz, T., Damjanović, A., Autenrieth, F., and Schulten, K. (2002). Photosynthetic apparatus of purple bacteria. *Quarterly reviews of biophysics*, 35. 1-62.

Hung, C.-H., Chang, Y.-T., and Chang, Y.-J. (2011). Roles of microorganisms other than *Clostridium* and *Enterobacter* in anaerobic fermentative biohydrogen production systems – A review. *Bioresource Technology*, 102(18), 8437-8444.

Huntley, M. E., and Redalje, D. G. (2007). CO2 mitigation and renewable oil from photosynthetic microbes: A new appraisal. *Mitigation and Adaptation Strategies for Global Change*, 12.573–608.

Huse, S.M. Welch, D. B. M.,Voorhis, A., Shipunova, A, Morrison, H. G., Eren, A. M. and Sogin, M. L.. (2014). VAMPS: a website for visualization and analysis of microbial population structures. *BMC Bioinformatics*, 15(41)

Idi, A., Ibrahim, Z., Mohamad, S. E., and Majid, Z. A. (2015). Biokinetics of nitrogen removal at high concentrations by *Rhodobacter sphaeroides* ADZ101. *International Biodeterioration & Biodegradation*, 105, 245–251.

Jamali, N. S. and Md Jahim, J. (2016). Optimization of Thermophilic Biohydrogen Production By Microflora of Palm Oil Mill Effluent: Cell Attachment on Granular Activated Carbon As Support Media. *Malaysian Journal of Analytical Science*, 20(6), 1437–1446.

Jamali, N. S., Jahim, J. M., Isahak, W. N. R. W., and Abdul, P. M. (2016). Particle size variations of activated carbon on biofilm formation in thermophilic biohydrogen production from palm oil mill effluent. *Energy Conversion and Management*, (September), 26–28.

Jamil, Z. Mohamad Annuar, M. S., Ibrahim, S. andVikineswary, S., (2009). Optimization of phototrophic hydrogen production by *Rhodopseudomonas palustris* PBUM001 via statistical experimental design. *International Journal of Hydrogen Energy*, 34(17), pp.7502–7512

Jessen, J. E. (2013). Biofuel Production from Lignocellulosic Biomass by Thermophilic Bacteria,PhD Thesis. University Akuriyei, Akuriyei.

Jessen, J. E., Sveinsson, T., Scully, S. M., and Orlygsson, J. (2015). Ethanol production by a *Paenibacillus* species isolated from an Icelandic hot spring – Production yields from complex biomass. Icelandic Agricultural Sciences, 28(1), 15-24.

Jin, P., Wang, B., Jiao, D., Sun, G., Wang, B., and Wang, X. C. (2015). Characterization of microflora and transformation of organic matters in urban sewer system. *Water Research*,84, 112–119.

Jun, Y.-S., Yu, S., Ryu, K., and Lee, T. (2008). Kinetic Study of pH Effects on Biological Hydrogen Production by a Mixed Culture. *Journal of Microbiology and Biotechnology*, 18, 1130–1135.

Juneja, V. K., Melendres, M. V., Huang, L., Subbiah, J., and Thippareddi, H. (2009). Mathematical modeling of growth of *Salmonella* in raw ground beef under isothermal conditions from 10 to 45 °C. *International Journal of Food Microbiology*, 131(2–3), 106–111.

Kalil, M. S., Alshiyab, H. S., Mohtar, W., Yusoff, W., and Selangor, B. (2008). Effect of Nitrogen Source and Carbon to Nitrogen Ratio on Hydrogen Production using *C . acetobutylicum. American Journal of Biochemistry and Biotechnology*, 4(4), 393–401.

Karadag, D., Mäkinen, A. E., Efimova, E., and Puhakka, J. A. (2009). Thermophilic biohydrogen production by an anaerobic heat treated-hot spring culture. *Bioresource Technology*, 100(23), 5790–5795.

Karadag, D. and Puhakka, J.A., (2010). Effect of changing temperature on anaerobic hydrogen production and microbial community composition in an open-mixed culture bioreactor. *International Journal of Hydrogen Energy*, 35(20), 10954-10959.

Kersters, K., De Vos, P., Gillis, M., Swings, J., Vandamme, P., and Stakebrandt, E. (2006). Introduction to the Proteobacteria. *Prokaryotes*,5. 3-37

Kim, D.-H., and Kim, M.-S. (2011). Hydrogenases for biological hydrogen production. *Bioresource Technology*, 102(18), 8423–8431.

Kim, D. H., and Kim, M. S. (2012). Thermophilic fermentative hydrogen production from various carbon sources by anaerobic mixed cultures. *International Journal of Hydrogen Energy*, 37(2), 2021–2027.

Kim, M. S., Kim, D. H., and Cha, J. (2012a). Culture conditions affecting Hydrogen production by phototrophic bacterium *Rhodobacter sphaeroides* KD131. *International Journal of Hydrogen Energy*, 37(19), 14055–14061.

Kim, M. S., Kim, D. H., Cha, J., and Lee, J. K. (2012b). Effect of carbon and nitrogen sources on photo-fermentative H_2 production associated with nitrogenase, uptake hydrogenase activity, and PHB accumulation in *Rhodobacter sphaeroides* KD131. *Bioresource Technology*, 116, 179–183.

King, R. L., and Botte, G. G. (2011). Hydrogen production via urea electrolysis using a gel electrolyte. *Journal of Power Sources*, 196(5), 2773–2778.

Koku, H. (2002). Aspects of the metabolism of hydrogen production by *Rhodobacter sphaeroides. International Journal of Hydrogen Energy*.27. 1315–1329

Koku, H. (2003). Kinetics of biological hydrogen production by the photosynthetic

bacterium *Rhodobacter sphaeroides* O.U. 001. *International Journal of Hydrogen Energy*, 28(4),381–388.

Kondo, T., Arakawa, M., Hirai, T., Tatsuki Wakayama, G., Hara, M., and Miyake, J. (2002). Enhancement of Hydrogen Production by a Photosynthetic Bacterium Mutant with Reduced Pigment. *Journal of Bioscience And Bioengineering*, 93(2). 145-150

Kuchenreuther, J. M., GradySmith, C. S., Bingham, A. S., George, S. J., Cramer, S. P., and Swartz, J. R. (2010). HighYield Expression of Heterologous [FeFe] Hydrogenases in *Escherichia coli. PLoS ONE*, 5(11), 2–7.

Kuczynsk, J, Stombaug, J., Walters, W. A., Gonzalez, A., Caporaso, G. and Knight, R.,(2012). Using QIIME to analyze 16S rRNA gene sequences from microbial communities. *Current Protocols in Bioinformatics*, (June),1–28

Lakshmi, K. V. N. S., Sasikala, C., and Ramana, C. V. (2009). *Rhodoplanes pokkaliisoli* sp. nov., a phototrophic alphaproteobacterium isolated from a water logged brackish paddy soil. *International Journal of Systematic and Evolutionary Microbiology*, 59(9), 2153–2157.

Lal, S., Romano, S., Chiarini, L., Signorini, A., and Tabacchioni, S. (2012). The *Paenibacillus polymyxa* species is abundant among hydrogen-producing facultative anaerobic bacteria in Lake Averno sediment. *Archives of Microbiology*, 194(5), 345–351.

Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., and Huttenhower, C. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology*, 31(9), 814–821.

Laocharoen, S., and Reungsang, A. (2014). Isolation, characterization and optimization of photo hydrogen production conditions by newly isolated *Rhodobacter sphaeroides* KKU-PS5. *International Journal of Hydrogen Energy*, 39(21), 10870–10882.

Laothanachareon, T., Kanchanasuta, S., Mhuanthong, W., Phalakornkule, C., Pisutpaisal, N., and Champreda, V. (2014). Analysis of microbial community adaptation in mesophilic hydrogen fermentation from food waste by tagged 16S rRNA gene pyrosequencing. *Journal of Environmental Management*, 144, 143–151.

Larsen, K. L., and Cox, R. P. (1996). Spectrochromatography of photosynthetic pigments as a fingerprinting technique for microbial phototrophs. *FEMS Microbiology Ecology*, 20, 69-77.

Lazaro, C. Z., Varesche, M. B. A., and Silva, E. L. (2015). Effect of inoculum concentration, pH, light intensity and lighting regime on hydrogen production by phototrophic microbial consortium. *Renewable Energy*, 75, 1–7.

Lazaro, C. Z., Vich, D. V., Hirasawa, J. S., and Varesche, M. B. A. (2012). Hydrogen production and consumption of organic acids by a phototrophic microbial consortium. *International Journal of Hydrogen Energy*, 37(16), 11691–11700.

Lee, K. S., Hsu, Y. F., Lo, Y. C., Lin, P. J., Lin, C. Y., and Chang, J. S. (2008). Exploring optimal environmental factors for fermentative hydrogen production from starch using mixed anaerobic microflora. *International Journal of Hydrogen Energy*, 33(5), 1565–1572.

Li, R. Y., and Fang, H. H. P. (2008). Hydrogen production characteristics of photoheterotrophic *Rubrivivax gelatinosus* L31. *International Journal of Hydrogen Energy*, 33(3), 974–980.

Li, X., Wang, Y., Zhang, S., Chu, J., Zhang, M., Huang, M., and Zhuang, Y. (2011). Effects of light/dark cycle, mixing pattern and partial pressure of Hydrogen on biohydrogen production by *Rhodobacter sphaeroides* ZX-5. *Bioresource Technology*, 102(2), 1142–8.

Lin, C. Y., Chang, C. C., and Hung, C. H. (2008a). Fermentative hydrogen production from starch using natural mixed cultures. *International Journal of Hydrogen Energy*, 33(10), 2445 2453.

Lin, C. Y., Wu, C. C., Wu, J. H., and Chang, F. Y. (2008b). Effect of cultivation temperature on fermentative hydrogen production from xylose by a mixed culture. *Biomass and Bioenergy*, 32(12), 1109–1115.

Liu, H., Zhang, T., and Fang, H. H. P. (2003). Thermophilic Hydrogen production from a cellulose containing wastewater. *Biotechnology Letters*, 25(4), 365-369.

Liu, B., Zhang, F., Feng, X., Liu, Y., Yan, X., Zhang, X., and Zhao, L. (2006). *Thauera* and *Azoarcus* as functionally important genera in a denitrifying quinolone-removal bioreactor as revealed by microbial community structure comparison. *FEMS Microbiology Ecology*, 55(2), 274–286.

Liu, B. F., Ren, N. Q., Tang, J., Ding, J., Liu, W. Z., Xu, J. F., and Xie, G. J. (2010). Bio-hydrogen production by mixed culture of photo- and dark-fermentation bacteria. *International Journal of Hydrogen Energy*, 35(7), 2858–2862.

Liu, J., Mbadinga, S. M., Ke, W., Gu, J., and Mu, B. (2016a). The diversity of hydrogen-producing microorganisms in a high temperature oil reservoir and its potential role in promoting the in situ bioprocess. *Applied Environmental Biotechnology*, 1(2).1-10

Liu, Y., Wan, J., Han, S., Zhang, S., and Luo, G. (2016b). Selective conversion of carbon monoxide to hydrogen by anaerobic mixed culture. *Bioresource Technology*, 202, 1–7.

Lo, Y. C., Chen, W. M., Hung, C. H., Chen, S. Der, and Chang, J. S. (2008). Dark hydrogen fermentation from sucrose and xylose using Hydrogen-producing indigenous bacteria: Feasibility and kinetic studies. *Water Research*, 42(4-5), 827–842.

Loss, R. A., Fontes, M. L., Reginatto, V., and Antônio, R. V. (2013). Biohydrogen production by a mixed photoheterotrophic culture obtained from a Winogradsky column prepared from the sediment of a southern Brazilian lagoon. *Renewable Energy*, 50, 648–654.

Ma, C., Wang, X., Guo, L., Wu, X., and Yang, H. (2012). Enhanced photofermentative hydrogen production by *Rhodobacter capsulatus* with pigment content manipulation. *Bioresource Technology*, 118, 490–5.

Madigan, M. T., and Gest, H. (1979). Growth of the photosynthetic bacterium *Rhodopseudomonas capsulata* chemoautotrophically in darkness with H_2 as the Energy Source. *Journal of Bacteriology*. 137(1):524-530

Maintinguer, S., Fernandes, B., Duarte, I., Saavedra, N., Adorno, M., and Varesche, M. (2008). Fermentative hydrogen production by microbial consortium. *International Journal of Hydrogen Energy*, 33(16), 4309–4317.

Maintinguer, S. I., Sakamoto, I. K., Adorno, M. A. T., and Varesche, M. B. A. (2015). Bacterial diversity from environmental sample applied to bio-hydrogen production. *International Journal of Hydrogen Energy*, 40(8), 3180–3190.

Mäkinen, A. E., Nissilä, M. E., and Puhakka, J. A. (2012). Dark fermentative hydrogen production from xylose by a hot spring enrichment culture. *International Journal of Hydrogen Energy*, 37(17), 12234–12240.

Manish, S., and Banerjee, R. (2008). Comparison of biohydrogen production processes. *International Journal of Hydrogen Energy*, 33(1), 279–286.

Mao, Y., Xia, Y., and Zhang, T. (2013). Characterization of *Thauera*-dominated hydrogen oxidizing autotrophic denitrifying microbial communities by using high-throughput sequencing. *Bioresource Technology*, 128, 703–710.

Mathur, J., Bizzoco, R.W., Ellis, D. G., Lipson, D. A., Poole, A. W., Levine, R. and Kelley, S.T. (2007). Effects of abiotic factors on the phylogenetic diversity of bacterial communities in acidic thermal springs. *Applied and Environmental Microbiology*, 73(8).2612–2623.

McEwan, a. G. (1994). Photosynthetic electron transport and anaerobic metabolism in purple non sulfur phototrophic bacteria. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, 66(1993), 151-164.

McFadden, B. a. (1973). Autotrophic CO_2 assimilation and the evolution of ribulose diphosphate carboxylase. *Bacteriological Reviews*, 37(3), 289–319.

McKinlay, J. B., and Harwood, C. S. (2010a). Carbon dioxide fixation as a central redox cofactor recycling mechanism in bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 11669–11675.

McKinlay, J. B., and Harwood, C. S. (2010b). Photobiological production of hydrogen gas as a biofuel. *Current Opinion in Biotechnology*, 21(3), 244–251.

Md Jahim, J. (2016). Optimization of Thermophilic Biohydrogen Production by Microflora of Palm Oil Mill Effluent: Cell Attachment on Granular Activated Carbon As Support Media. *Malaysian Journal of Analytical Science*, 20(6), 1437–1446.

Melnicki, M., Bianchi, L., Dephilippis, R., and Melis, A. (2008). Hydrogen production during stationary phase in purple photosynthetic bacteria. *International Journal of Hydrogen Energy*, 33(22), 6525–6534.

Meyer, F. Paarmann, D., Souza, M. D., Olson, R., Glass, E. M., Kubal, M., Paczian, T., Rodriguez, A., Stevens, R.,Wilke, A., Wilkening, J., and Edwards, R. A.,(2008). The Metagenomics RAST Server: A Public Resource for the Automatic Phylogenetic and Functional Analysis of Metagenomes. *BMC Bioinformatics*, 9(386), 1–8.

Minnan, L., Jinli, H., Xiaobin, W., Huijuan, X., Jinzao, C., Chuannan, L., and Liangshu, X. (2005). Isolation and characterization of a high H_2-producing

strain *Klebsiella oxytoca* HP1 from a hot spring. *Research in Microbiology*, 156(1), 76–81.

Mishra, P., Roy, S., and Das, D. (2015). Comparative evaluation of the hydrogen production by mixed consortium, synthetic co-culture and pure culture using distillery effluent. *Bioresource Technology*, 198, 593–602.

Mohd Ghazali, S.A. and Abdul Hamid, T.H.T., 2015. New lipase producing β-proteobacteria strains *Caldimonas* sp. and *Tepidimonas* sp. isolated from a Malaysian hot springs. *Sains Malaysiana*, 44(5), 701–706.

Mohd Yasin, N.H., Abd Rahman, N. A., Che Man, H., Mohd Yusoff, M. Z., and Hassan, M. A. (2011). Microbial characterization of hydrogen-producing bacteria in fermented food waste at different pH values. *International Journal of Hydrogen Energy*, 36(16) 9571–9580.

Mohd Yasin, N.H., Fukuzaki, M., Maeda, T., Miyazaki, T., Mohd, C. C., Hakiman Ariffin, H. and Wood, T. K. (2013). Biohydrogen production from oil palm frond juice and sewage sludge by a metabolically engineered *Escherichia coli* strain. *International Journal of Hydrogen Energy*, 38(25), 10277–10283.

Montiel-Corona, V., Revah, S. and Morales, M. (2015). Hydrogen production by an enriched photoheterotrophic culture using dark fermentation effluent as substrate: Effect of flushing method, bicarbonate addition, and outdoor–indoor conditions. *International Journal of Hydrogen Energy*, 40(30) 9096–9105.

Morimoto, M. (2004). Biological production of hydrogen from glucose by natural anaerobic microflora. *International Journal of Hydrogen Energy*, 29(7), 709-713.

Morimoto, K., Kimura, T., Sakka, K., Ohmiya, K. (2005). Overexpression of a hydrogenase gene in Clostridium paraputrificum to enhance hydrogen gas production. *FEMS Microbiology Letters*, 246(2).229–234.

Mortenson, L. E., Schlegel, H. G., Schneider, K., Activity, C., Bothe, H., Eisbrenner, G., and Chen, J. S. (1981). Hydrogen production by photosynthetic bacteria. *Experentia*, 64–66.

Mu, Y., Wang, G., and Yu, H. Q. (2006). Kinetic modeling of batch hydrogen production process by mixed anaerobic cultures. *Bioresource Technology*, 97(11), 1302–1307.

Mu, Y., Yu, H.-Q., and Wang, G. (2007). A kinetic approach to anaerobic hydrogen producing process. *Water Research*, 41(5), 1152–1160.

Mu, Y., Yang, H.-Y., Wang, Y.-Z., He, C.-S., Zhao, Q.-B., Wang, Y., and Yu, H.-Q. (2014). The maximum specific hydrogen-producing activity of anaerobic mixed cultures: definition and determination. *Scientific Reports*, 4(January), 5239.

Muyzer, G., and Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek*, 73, 127–141.

Muyzer, G. (1999). DGGE/TGGE a method for identifying genes from natural ecosystems.*Current Opinion in Microbiology*, 2(3), 317–322.

Nandi, R., and Sengupta, S. (1998). Microbial Production of Hydrogen: An Overview. *Critical Reviews in Microbiology*, 24(1), 61–84.

Nath, K., Muthukumar, M., Kumar, A. and Das, D. (2008). Kinetics of two-stage fermentation process for the production of hydrogen. *International Journal of Hydrogen Energy*, 33(4)1195–1203.

Nath, K., and Das, D. (2011). Modeling and optimization of fermentative hydrogen production. *Bioresource Technology*, 102(18), 8569–81.

Nelson, D., and Cox, M. (2008). Lehninger: Principles of Biochemistry - Fifth edition. New York: W.H. Freeman and Company.

Nguimkeu, P. (2014). A simple selection test between the Gompertz and Logistic growth models. *Technological Forecasting and Social Change*, 88(1), 98–105.

Nilakanta, H., Drews, K. L., Firrell, S., Foulkes, M. A., and Jablonski, K. A. (2014). A review of software for analyzing molecular Sequences. *BMC Research Notes*, 7(1)

Norashirene, M. J., Umi Sarah, H., K., Siti, M. H., and Nurdiana, S. (2013). Biochemical Characterization and 16S rDNA Sequencing of Lipolytic Thermophiles from Selayang Hot Spring, Malaysia. *IERI Procedia*, 5, 258-264.

O-Thong, S., Prasertsan, P., Karakashev, D., and Angelidaki, I. (2008). 16S rRNA-targeted probes for specific detection of *Thermoanaerobacterium* spp., *Thermoanaerobacterium thermosaccharolyticum*, and *Caldicellulosiruptor* spp. by fluorescent in situ hybridization in biohydrogen producing systems. *International Journal of Hydrogen Energy*, 33(21), 6082–6091.

Ottaviano, L. M., Ramos, L. R., Botta, L. S., Amâncio Varesche, M. B., and Silva, E. L. (2016). Continuous thermophilic hydrogen production from cheese whey

powder solution in an anaerobic fluidized bed reactor: Effect of hydraulic retention time and initial substrate concentration. *International Journal of Hydrogen Energy*, 2 (42). 4848-4860

Pandey, A., Srivastava, N., and Sinha, P. (2012). Optimization of hydrogen production by *Rhodobacter sphaeroides* NMBL-01. *Biomass and Bioenergy*, 37, 251–256.

Park, K. S., Ki, C.-S., Kang, C.-I., Kim, Y.-J., Chung, D. R., Peck, K. R., and Lee, N. Y. (2012). Evaluation of the GenBank, EzTaxon, and BIBI services for molecular identification of clinical blood culture isolates that were unidentifiable or misidentified by conventional methods. *Journal of Clinical Microbiology*, 50(5), 1792–5.

Park, J., Lee, S., Ju, H., Kim, S., Yoon, J., and Park, H. (2016). Failure of biohydrogen production by low levels of substrate and lactic acid accumulation. *Renewable Energy*, 86, 889–894.

Pawar, S. S., and van Niel, E. W. J. (2013). Thermophilic biohydrogen production: how far are we? Applied Microbiology and Biotechnology, 97(18), 7999-8009.

Pfennig, N. (1969). *Rhodopseudomonas acidophila* , sp . n., a New Species of the Budding Purple Nonsulfur Bacteria. *Journal of Bacteriology*, 99(2), 597–602.

Phowan, P., and Danvirutai, P. (2014). Hydrogen production from cassava pulp hydrolysate by mixed seed cultures: Effects of initial pH, substrate and biomass concentrations. *Biomass and Bioenergy*, 64, 1–10.

Phummala, K., Imai, T., Reungsang, A., Chairattanamanokorn, P., Sekine, M., Higuchi, T., and Kanno, A. (2014). Delignification of disposable wooden chopsticks waste for fermentative hydrogen production by an enriched culture from a hot spring. *Journal of Environmental Sciences (China)*, 26(6), 1361-1368.

Plummer, E. and Twin, J. (2015). A Comparison of Three Bioinformatics Pipelines for the Analysis of Preterm Gut Microbiota using 16S rRNA Gene Sequencing Data. *Journal of Proteomics & Bioinformatics*, 8(12) 283–291.

Portillo, M.C., Sririn, V., Kanoksilapathanm, W. and Gonzalez, J. M. 2009. Differential microbial communities in hot spring mats from Western Thailand. *Extremophiles*, (13) 321–331.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., Glöckner, F. O. (2007). SILVA: A comprehensive online resource for quality checked and

aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research*, 35(21)7188–7196.

Puhakka, J. A., Karadag, D., and Nissilä, M. E. (2012). Comparison of mesophilic and thermophilic anaerobic hydrogen production by hot spring enrichment culture. *International Journal of Hydrogen Energy*, 37(21), 16453–16459.

Rasdi, Z., Abdul Rahman, N. A., Abd-aziz, S., Lai-yee, P., Mohd Yusoff, M. Z., Mei-ling, C. Hassan, M. A. (2009). Statistical Optimization of Biohydrogen Production from Palm Oil Mill Effluent by Natural Microflora. *The Open Biotechnology Journal*, 3,79–86.

Rasdi, Z., Mumtaz, T., Abdul Rahman, N., and Hassan, M. A. (2012). Kinetic analysis of biohydrogen production from anaerobically treated POME in bioreactor under optimized condition. *International Journal of Hydrogen Energy*, 37(23), 17724–17730.

Ratti, R. P., Botta, L. S., Sakamoto, I. K., and Varesche, M. B. A. (2013). Microbial diversity of hydrogen-producing bacteria in batch reactors fed with cellulose using leachate as inoculum. *International Journal of Hydrogen Energy*, 38(23), 9707–9717.

Ratti, R. P., Delforno, T. P., Okada, D. Y., and Varesche, M. B. A. (2015). Bacterial communities in thermophilic Hydrogen-producing reactors investigated using 16S rRNA 454 pyrosequencing. *Microbiological Research*, 173, 10–17.

Ren, N.-Q., Liu, B.-F., Ding, J., and Xie, G.-J. (2009). Hydrogen production with *R. faecalis* RLD 53 isolated from freshwater pond sludge. *Bioresource Technology*, 100 (1). 484-487

Rettedal, E. A., Clay, S., and Brözel, V. S. (2010). GC-clamp primer batches yield 16S rRNA gene amplicon pools with variable GC clamps, affecting denaturing gradient gel electrophoresis profiles. *FEMS Microbiology Letters*, 312(1), 55-62.

Rey, F. E., Heiniger, E. K., and Harwood, C. S. (2007). Redirection of metabolism for biological hydrogen production. *Applied and Environmental Microbiology*, 73(5), 1665–1671.

Riehle, K., Coarfa, C., Jackson, A., Ma, J., Tandon, A., Paithankar, S., Raghuraman, S., Mistretta, T. A., Saulnier, D., Raza, S., Diaz, M. A., Shulman, R., Aagaard, K., Versalovic, J., and Milosavljevic, A. (2012). The Genboree Microbiome

Toolset and the analysis of 16S rRNA microbial sequences. *BMC bioinformatics*, 13(S11) 1-11

Roy, S., Ghosh, S., and Das, D. (2012). Improvement of hydrogen production with thermophilic mixed culture from rice spent wash of distillery industry. *International Journal of Hydrogen Energy*, 37(21), 15867–15874.

Roy, S., Kumar, K., Ghosh, S., and Das, D. (2014). Thermophilic biohydrogen production using pre-treated algal biomass as substrate. *Biomass and Bioenergy*, 61, 157–166.

Rundell, E. A., Banta, L. M., Ward, D. V., Watts, C. D., Birren, B., and Esteban, D. J. (2014). 16S rRNA Gene Survey of Microbial Communities in winogradsky columns. *PLoS ONE*, 9(8).

Saady, N. M. C. (2013). Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge. *International Journal of Hydrogen Energy*, 38(30),13172–13191.

Sakaff, M. K. L. M., Rahman, A. Y. A., Saito, J. A., Hou, S., and Alam, M. (2012). Complete genome sequence of the thermophilic bacterium *Geobacillus thermoleovorans* CCB_US3_UF5. *Journal of Bacteriology*. 5. 1239-1239

Samsudin, A.R., Hamzah, U., Rahman, R. A., Siwar, C., Mohd Jani, M. F. and Othman, R. (1997). Thermal springs of Malaysia and their potential development. *Journal of Asian Earth Sciences*, 15(2–3), pp.275–284.

Savichtcheva, O., Joris, B., Wilmotte, A., and Calusinska, M. (2011). Novel FISH and quantitative PCR protocols to monitor artificial consortia composed of different hydrogen-producing *Clostridium* spp. *International Journal of Hydrogen Energy*, 36(13), 7530–7542.

Schiraldi, C. and De Rosa, M. (2002). The production of biocatalysts and biomolecules from extremophiles. *Trends in Biotechnology*, 20(12).515–521.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., and Weber, C. F. (2009). Introducing mothur: Open-source, platform-independent, community supported software for describing and comparing microbial communities.*Applied and Environmental Microbiology*, 75(23), 7537–7541.

Seifert, K. and Zagrodnik, R. (2009). Photofermentative Hydrogen Generation in Presence of Waste Water from Food Industry. Biogas. InTech.

Seifert, K., Waligorska, M., and Laniecki, M. (2010). Hydrogen generation in photobiological process from dairy wastewater. *International Journal of Hydrogen Energy*, 35(18), 9624-9629.

Sevinç, P., Gündüz, U., Eroglu, I., and Yücel, M. (2012). Kinetic analysis of photosynthetic growth, hydrogen production and dual substrate utilization by *Rhodobacter capsulatus. International Journal of Hydrogen Energy*, 37, 16430–16436.

Shanmugam, S. R., Rao, S., Lalman, J. A., and Heath, D. D. (2013). Effect of inhibitors on hydrogen consumption and microbial population dynamics in mixed anaerobic cultures. *International Journal of Hydrogen Energy*, 39(1), 249-257.

Shanmugam, S. R., Rao, S., Lalman, J. A., and Heath, D. D. (2014). Statistical Optimization of conditions for minimum Hydrogen consumption in mixed anaerobic cultures: Effect on homoacetogenesis and methanogenesis. *International Journal of Hydrogen Energy*, 39(28), 15433–15445.

Sharp, C.E., Brady, A. L., Sharp, G. H., Grasby, S. E., Stott, M. B. and Dunfield, P. F. (2014). Humboldt's spa: Microbial diversity is controlled by temperature in geothermal environments. *ISME Journal*, 8(6),1166–1174.

Sheffield, V. C., Cox, D. R., Lerman, L. S. and Myers, R. M. (1989). Attachment of a 40-base pair G + C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proceedings of the NationalAcademy of Sciences of the United States of America*, 86(1), 232–236.

Shin, H. S., Youn, J. H., and Kim, S. H. (2004). Hydrogen production from food waste in anaerobic mesophilic and thermophilic acidogenesis. *International Journal of Hydrogen Energy*, 29(13), 1355–1363.

Sikora, A., Błaszczyk, M., Jurkowski, M., and Zielenkiewicz, U. (2013). Lactic acid bacteria in hydrogen-producing consortia: on purpose or by coincidence? Lactic Acid Bacteria. *R&D for Food, Health and Livestock Purposes*, 487-514.

Singh, R. (2013). Fermentative Biohydrogen Production Using Microbial Consortia. In Biofuel Technologies (pp. 273–299). Berlin, Heidelberg: Springer Berlin Heidelberg.

Singh, L., Siddiqui, M. F., Ahmad, A., Rahim, M. H. A., Sakinah, M. and Wahid, Z. A. (2013). Biohydrogen production from palm oil mill effluent using

immobilized mixed culture. *Journal of Industrial and Engineering Chemistry*, 19(2) 659–664.

Singh, L., and Wahid, Z. A. (2015). Methods for enhancing bio-hydrogen production from biological process: A review. *Journal of Industrial and Engineering Chemistry*, 21, 70–80.

Sinha, P., and Pandey, A. (2011). An evaluative report and challenges for fermentative biohydrogen production. *International Journal of Hydrogen Energy*, 36(13), 7460–7478.

Sinha, P., Roy, S. and Das, D. (2016). Genomic and proteomic approaches for dark fermentative biohydrogen production. *Renewable and Sustainable Energy Reviews*, 56.1308–1321

Sirevåg, R., Buchanan, B. B., Berry, J. a., and Troughton, J. H. (1977). Mechanisms of CO_2 fixation in bacterial photosynthesis studied by the carbon isotope fractionation technique. *Archives of Microbiology*, 112, 35–38.

Sittijunda, S., and Reungsang, A. (2012). Biohydrogen production from waste glycerol and sludge by anaerobic mixed cultures. *International Journal of Hydrogen Energy*, 37(18), 13789-13796.

Sivagurunathan, P., Sen, B., and Lin, C. (2014a). Batch fermentative hydrogen production by enriched mixed culture: Combination strategy and their microbial composition. *Journal of Bioscience and Bioengineering*, 117(2), 222–228.

Sivagurunathan, P., Sen, B., and Lin, C. Y. (2014b). Overcoming propionic acid inhibition of hydrogen fermentation by temperature shift strategy. *International Journal of Hydrogen Energy*, 39(33), 19232–19241.

Sivagurunathan, P., Kumar, G., Park, J. H., Park, J. H., Park, H. D., Yoon, J. J., and Kim, S. H. (2016). Feasibility of enriched mixed cultures obtained by repeated batch transfer in continuous hydrogen fermentation. *International Journal of Hydrogen Energy*, 41(7), 4393–4403.

Smyth, T. J. P., Perfumo, A., McClean, S., Marchant, R., and Banat, I. M. (2010). Handbook of Hydrocarbon and Lipid Microbiology. Springer-Verlag Berlin, Heidelberg

Staley, J., Gunsalus, R., Lory, S., and Perry, J. (2007). Microbial life - 2nd edition. Sunderland, MA: Sinauer Associates Inc.

Stoppani, A. O. M., R. C. Fuller, and M. C. (1954). Carbon Dioxide Fixation By *Rhodopseudomonas capsulatus*, 69. 491–501.

Su, H., Cheng, J., Zhou, J., Song, W., and Cen, K. (2009). Combination of dark- and photo fermentation to enhance hydrogen production and energy conversion efficiency. *International Journal of Hydrogen Energy*, 34(21), 8846–8853.

Susana M. Coelho, Nathalie Simon, Sophia Ahmed, J. M. C. and F. P. (2013). Ecological and evolutionary genomics of marine photosynthetic organisms. *Molecular Ecology*, 867–907.

Tang, G.-L., Huang, J., Sun, Z.-J., Tang, Q.-Q., Yan, C.-H., and Liu, G.-Q. (2008). Biohydrogen production from cattle wastewater by enriched anaerobic mixed consortia: influence offermentation temperature and pH. *Journal of Bioscience and Bioengineering*, 106(1), 80-87.

Tapia-Venegas, E., Ramirez-Morales, J. E., Silva-Illanes, F., Toledo-Alarcón, J., Paillet, F., Escudie, R., Lay, C-H., Chu, C-H., Leu, H-J., Marone, A., Lin, C Y., Kim, D-H., Trably, E. and Ruiz-Filippi, G. (2015). Biohydrogen production by dark fermentation: scaling-up and technologies integration for a sustainable system. *Reviews in Environmental Science and BioTechnology*, 14(4) 761-785.

Timpmann, K., Chenchiliyan, M., Jalviste, E., Timney, J. a., Hunter, C. N., and Freiberg, A. (2014). Efficiency of light harvesting in a photosynthetic bacterium adapted to different levels of light. *Biochimica et Biophysica Acta Bioenergetics*, 1837(10), 1835–1846.

Tiwari, A., and Pandey, A. (2012). Cyanobacterial hydrogen production – A step towards clean environment. *International Journal of Hydrogen Energy*, 37(1), 139–150.

Tjørve, K. M. C., and Tjørve, E. (2017). The use of Gompertz models in growth analyses, and new Gompertz-model approach: An addition to the Unified-Richards family. *PLoS ONE*, 12(6), 1–17.

Uyar, B., Eroglu, I., Yücel, M., and Gündüz, U. (2009). Photofermentative hydrogen production from volatile fatty acids present in dark fermentation effluents. *International Journal of Hydrogen Energy*, 34(10), 4517–4523.

Uyar, B., Eroglu, I., Yucel, M., Gunduz, U., and Turker, L. (2007). Effect of light intensity, wavelength and illumination protocol on hydrogen production in photobioreactors. *International Journal of Hydrogen Energy*, 32(18), 4670-4677.

Valdez-Vazquez, I., and Poggi-Varaldo, H. M. (2009). Hydrogen production by fermentative consortia. *Renewable and Sustainable Energy Reviews*, 13(5), 1000–1013.

Vasconcelos, E. A. F., Leitao, R. C., and Santaella, S. T. (2016). Factors that affect bacterial ecology in hydrogen-producing anaerobic reactors. *Bioenergy Research*, 9(4), 1260–1271.

Venkata Mohan, S., Agarwal, L., Mohanakrishna, G., Srikanth, S., Kapley, A., Purohit, H. J., and Sarma, P. N. (2011). Firmicutes with iron dependent hydrogenase drive hydrogen production in anaerobic bioreactor using distillery wastewater. *International Journal of Hydrogen Energy*, 36(14),8234–8242.

Vijayaraghavan, K. and Ahmad, D., 2006. Biohydrogen generation from palm oil mill effluent using anaerobic contact filter. *International Journal of Hydrogen Energy*, 31(10) 1284–1291

Wahlund, T. M., Woese, C. R., Castenholz, R. W., and Madigan, M. T. (1991). A thermophilic green sulfur bacterium from New Zealand hot springs, *Chlorobium tepidum* sp. nov. *Archives of Microbiology*, 156, 81–90.

Wang, J., and Wan, W. (2008). Effect of temperature on fermentative hydrogen production by mixed cultures. *International Journal of Hydrogen Energy*, 33(20), 5392–5397.

Wang, X., Monis, P. T., Saint, C. P., and Jin, B. (2008). Biochemical kinetics of fermentative hydrogen production by *Clostridium butyricum* W5. *International Journal of Hydrogen Energy*, 34, 791–798.

Wang, J., and Wan, W. (2009a). Application of desirability function based on neural network for optimizing biohydrogen production process. *International Journal of Hydrogen Energy*, 34(3), 1253–1259.

Wang, J., and Wan, W. (2009b). Kinetic models for fermentative hydrogen production: A review. *International Journal of Hydrogen Energy*, 34(8), 3313–3323.

Wang, Y., and Qian, P.-Y. (2009). Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomics studies. *PloS One*, 4(10), 7401.

Wang, Y.-Z., Liao, Q., Zhu, X., Li, J., and Lee, D.-J. (2011). Effect of culture conditions on the kinetics of hydrogen production by photosynthetic bacteria in batch culture. *InternationalJournal of Hydrogen Energy*, 36(21), 14004-

14013.

Wang, S., Zhang, T., and Su, H. (2015). Enhanced hydrogen production from corn starch wastewater as nitrogen source by mixed cultures. *Renewable Energy*, 96.

Weaver, P. F., Wall, J. D., and Gest, H. (1975). Characterization of *Rhodopseudomonas capsulata. Archives of Microbiology*, 105, 207–216.

Weiland, P. (2010). Biogas production: Current state and perspectives. *Applied Microbiology and Biotechnology*, 85(4), 849–860.

Weisburg, W. G., Barns, S. M., Pelletier, D. a., and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, 173(2), 697–703.

Widdel, F., Boetius, A., and Rabus, R. (2006). Proteobacteria: Gamma Subclass. The Prokaryotes. A Handbook on the Biology of Bacteria. Springer. USA

Wirtz, M. and Hell, R. (2008). Metabolism of Cysteine in Plants and Phototrophic Bacteria. Sulfur metabolism in phototrophic organisms. Springer.USA

Wong, Y. M., Wu, T. Y., and Juan, J. C. (2014). A review of sustainable hydrogen production using seed sludge via dark fermentation. *Renewable and Sustainable Energy Reviews*, 34, 471–482.

Wongthanate, J., Chinnacotpong, K., and Khumpong, M. (2014). Impacts of pH, temperature and pretreatment method on biohydrogen production from organic wastes by sewage microflora. *International Journal of Energy and Environmental Engineering*, 5(1), 1–6.

Xing, D., Cheng, S., Regan, J. M., and Logan, B. E. (2009). Change in microbial communities in acetate- and glucose-fed microbial fuel cells in the presence of light. *Biosensors & Bioelectronics*, 25(1), 105–11.

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(18), 4761–4771.

Yanling, Y., Zhenmei, Y.L., Hang, M. and Jun C. (2008). Dynamic changes of microbial community diversity in a photohydrogen producing reactor monitored by PCR-DDGE. *Journal of Environmental Science*, 20,1118–1125.

Yingli, R., Zhang, T., and Fang, H. (2008). Characteristics of a phototrophic sludge producing hydrogen from acetate and butyrate. *International Journal of Hydrogen Energy*, 33(9), 2147–2155.

Yoon, S. H., Ha, S. M., Kwon, S., Lim, J., Kim, Y., Seo, H., and Chun, J. (2017). Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole genome assemblies. *International Journal of Systematic and Evolutionary Microbiology*, 67(5), 1613–1617.

Yossan, S., O-Thong, S.and Prasertsan, P., (2012). Effect of initial pH, nutrients and temperature on hydrogen production from palm oil mill effluent using thermotolerant consortia and corresponding microbial communities. *International Journal of Hydrogen Energy*, 37(18)13806–13814.

Yurkov, V. V, and Beatty, J. T. (1998). Aerobic anoxygenic phototrophic bacteria. *Microbiology and Molecular Biology Reviews: MMBR*, 62(3), 695–724.

Yusoff, M.Z.M., Hassan, M., Abd-Aziz, S. and Rahman, N. A. A. (2009). Start-Up of biohydrogen production from palm oil mill effluent under non-sterile condition in 50 L continuous stirred tank reactor. *International Journal of Agricultural Research*, 4(4) 163–168.

Zagrodnik, R., and Laniecki, M. (2015). The role of pH control on biohydrogen production by single stage hybrid dark- and photo-fermentation. *Bioresource Technology*, 194, 187–95.

Zahoor, S., Javed, M. M., and Aftab, M. N. (2012). Isolation and Molecular Identification of a Facultatively Anaerobic Bacterium from the Hot Spring Of Azad Kashmir. *Pakistan Journal of Botany*, (March), 329–333.

Zhang, T. and Fang, H.H.P. (2000). Digitization of DGGE (denaturing gradient gel electrophoresis) profile and cluster analysis of microbial communities. *Environmental Engineering*, 22(5), 399–405.

Zhang, T., Liu, H. and Fang, H.H.P., (2003). Biohydrogen production from starch in wastewater under thermophilic condition. , 69.149–156.

Zhang, Y. and Shen, J. (2006). Effect of temperature and iron concentration on the growth and hydrogen production of mixed bacteria. *International Journal of Hydrogen Energy*, 31(4)441–446.

Zhang, F., Chen, Y., Dai, K., Shen, N., and Zeng, R. J. (2015). The glucose metabolic distribution in thermophilic (55 °C) mixed culture fermentation: A chemostat study. *International Journal of Hydrogen Energy*, 40(2), 919–926.

Zhang, F., Yang, J., Dai, K., and Ding, Z. (2016a). Microbial dynamics of the extreme thermophilic (70° C) mixed culture for hydrogen production in a chemostat. *International Journal of Hydrogen Energy*, 41(26), 11072–11080.

Zhang, F., Yang, J. H., Dai, K., Chen, Y., Li, Q. R., Gao, F. M., and Zeng, R. J. (2016b). Characterization of microbial compositions in a thermophilic chemostat of mixed culture fermentation. *Applied Microbiology and Biotechnology*, 100(3), 1511–15

Zwietering, M.H., Jongenburger, I., Rombouts, F. M. and Van't Riet, K. (1990). Modeling of the bacterial growth curve. *Applied and Environmental Microbiology*, 56(6), pp.1875–1881.

Appendix C

Promega Wizard® Genomic DNA Purification kit Protocol

1 mL of the overnight culture was centrifuged at 16 0000 x g for two minutes to obtain cell pellet. This step was repeated about five times with the overnight culture to obtain higher amount of pellet. Then, the cells were suspended thoroughly in 480 µl of 50 mM EDTA followed by addition of 120 µL of lysozyme and was mixed gently. The puroose of this pre-treatment was to weaken the cell wall so that cell lysis could efficiently took place. Next, the sample was incubated at 37°C for 60 minutes on the Thermomixer® (Eppendorf) and then was centrifuged for 2 minutes at 16, 000 x g. After that, the supernatant was discarded. Then, 600 µL of Nuclei Lysis solution was added and mix gently.

Next, the cells were lysed by incubation at 80°C on the Thermomixer® (Eppendorf) for 5 minutes and then was cooled at room temperature. Three µl of RNAse solution was added to the cell lysate and the tube was inverted 2-5 times to mix. After that, the tube was incubated at 37°C for 60 minutes before it was cooled at room temperature. Then, 200 µL Protein Precipitation Solution was added to the RNAse-treated cell lysate and then was vortexed vigorously at high speed for 20 seconds. Next, the sample was incubated for 5 minutes on ice and followed by centrifugation at 16, 000 x g for 3 minutes.

Then, the supernatant containing the DNA was carefully transferred to a clean 1.5 mL microcentrifuge tube containing 600 µL of room temperature isopropanol. The supernatant was carefully transferred without any contamination of the pellet which is the precipitated protein. The tube containing the mixture was gently inverted until a thread-like strands of DNA form a visible mass. The mixture was centrifuged at 16, 000 x g for 2 minutes. Then, carefully the supernatant was poured off and drained from the tube on clean absorbent paper. After that, about 600 µL 70% (v/v) ethanol was added and the tube was gently inverted several times to wash the DNA pellet. Again, the tube was centrifuged at the same power for 2 minutes and excess ethanol was carefully aspirated. The tube was drained on a clean absorbent paper and the pellet was

allowed to air dry for 10 to 15 minutes. Then, 50 µL of DNA rehydration solution was added to the tube and the DNA was rehydrated by incubating at 65°C for 1 hour on the Thermomixer® (Eppendorf). The solution was periodically mixed by gently tapping the tube. Alternatively, the DNA was rehydrated by incubating the solution overnight at 4°C. Finally, the DNA was stored at 2-8°C to use as a working solution or was stored at - 20°C for longer storage.