

BIOHYDROGEN PRODUCTION BY A MICROBIAL CONSORTIUM ISOLATED FROM
LOCAL HOT SPRING

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BIOHYDROGEN PRODUCTION BY A MICROBIAL CONSORTIUM
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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

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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 H₂/L culture with the maximal production rate of 4.117 mmol/L h⁻¹, biohydrogen yield obtained was 135.7 mmol H₂/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 keupayaannya 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 polimerase 16S rRNA-gel elektroforesis penyahasian 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 H₂/L dengan kadar penghasilan maksimum 4.117 mmol/L h⁻¹, hasil biohidrogen adalah sebanyak 135.7 mmol H₂/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.

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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
T _m	-	melting point
V	-	volt

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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 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 (Gadamshetty *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 hypothesized 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,

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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 purpose of this pre-treatment was to weaken the cell wall so that cell lysis could efficiently take 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 mixed 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 $2-8^{\circ}\text{C}$ to use as a working solution or was stored at -20°C for longer storage.