Doctoral Dissertation

A study of biohydrogen production by extremely halophilic bacterial communities from a salt pan and salt damaged soil

(塩田と塩害土壌から得られた高度塩分耐性菌叢による
バイオ水素生産に関する研究)

March 2020

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ABSTRACT

The world's energy today is still relying heavily on fossil fuel. The carbon-containing oil, coal, and natural gas are consumed rapidly over the past century, leading to an accelerated release of carbon dioxide. It has been a consensus that the increasing CO_2 concentration in the earth's atmosphere is a major cause of global warming and the related climate change. As the Intergovernmental Panel on Climate Change is setting the target to lower the global net human-caused emissions of carbon dioxide by about 45% by 2030 from the 2010 levels and set the goal of "net zero" in 2050, drastic measures are needed to reach these goals.

One of the options is by increasing the efficiency of electricity production and energy use in transportation and industry. Another available option is through the development of alternative energy sources, such as hydrogen. Currently, hydrogen is still mainly produced through steam-reforming of natural gas. A shift to hydrogen production from renewable sources will help to relieve the world's dependency on fossil fuel. One of the available options is through biological hydrogen production from lignocellulosic biomass.

The application of extremely halophilic bacteria for biohydrogen production from lignocellulosic biomass can potentially contribute to a cost reduction of sterilization and water. While lignocellulosic biomass is available in great quantity in nature from woods, grasses, and agricultural wastes, it has a naturally recalcitrant structure of cellulose, hemicellulose, and lignin. The cellulose and hemicellulose part can be biologically utilized through the fermentation process after the application of size reduction, pretreatment, and enzymatic saccharification. Alkaline pretreatment with NaOH is considered as the most widely used and cost-effective for lignocellulosic biomass. By using extremely halophilic bacteria, after lignocellulosic pretreatment with NaOH and neutralization with HCl, only a relatively small amount of water will be needed to dilute the pretreated biomass before the fermentation process.,

Extremely halophilic hydrogen-producing bacteria were investigated, owing to their ability to live in high-salinity conditions. Based on this characteristic, it was hypothesized that extremely halotolerant hydrogen-producing bacteria could tolerate high concentrations of Na^+ ions. To test this hypothesis, we investigated the characteristics of extremely halotolerant hydrogen-producing bacteria obtained from salt-damaged soil in Khon Kaen and a commercial salt pan field near Bangkok (Samut Sakhon), Thailand. The result of this preliminary investigation showed that hydrogen production under saturated conditions of 26% (6 M) NaCl was possible after one year of acclimatization.

The optimum conditions for biohydrogen production from glucose with extremely halophilic bacteria from Samut Sakhon salt pan, Thailand, were then identified. A hydrogen molar yield of 1.45 mol H_2/mol glucose at optimum conditions of 26% NaCl, 35°C, and pH 9 was obtained. The acclimatized bacteria were able to ferment various types of lignocellulosic-derived sugars and D-fructose, which also suggested a potential ability to produce biohydrogen from food waste. Through PCR-DGGE, the predominant hydrogen producer among the mixed culture was found to be *Halanaerobium fermentans*. A new 16s rRNA sequence of *H. fermentans* strain B4 has been identified and submitted to the GenBank data base (Accession number MN133965). The 1,424 base-pair length sequence shares 99.36% similarity with strain R-9, which has been isolated from salted puffer fish ovaries in Japan and reported in 2000. These findings provided insights into the application of extremely halophilic bacteria for biohydrogen production.

A study of three extreme halophilic microbial communities cultivated from the soils of a salt pan and salt damaged soil in Thailand was also presented. The cultivation aimed to screen potential biohydrogen producing bacteria capable of growing in 26% NaCl concentration. Polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) of the V3 region of the 16s rRNA and the high-throughput amplicon sequencing of the V3-V4 regions of 16s rRNA performed on IonS5™XL platform techniques were used, and the results were compared. Several bands corresponded to the same genus of *Halanaerobium* in the DGGE result, limiting the technique to provide only a rough idea of the profiles of extreme halophilic bacterial communities. High-throughput amplicon sequencing revealed that the most abundant OTU among all communities (37-98%) shared close similarity with *Halanaerobium fermentans* R-9, while one community has many highly similar OTUs to *Halanaerobacter lacuranum* TB21 (61%). Higher hydrogen molar yields were obtained by microbial communities with the higher population of *H. fermentans* (1.15 and 1.08 mol H_2/mol glucose) compared to microbial communities dominated by H. *lacunarum* (0.66 mol H_2/mol glucose). The amplicon sequencing with NGS has provided more detailed pictures compared to the PCR-DGGE technique for the extreme halophilic bacterial communities.

概要

世界のエネルギー事情は、現在でも化石燃料に頼り切っている。炭素を含有したオイル、石炭、そして天然ガスはこの 1 世紀の間に急激に消費され、それが二酸化炭素の排出に拍車をかけている。大気中の二酸化炭素濃度の増加が地球温暖化や気候変動の主な原因であるということは一般的に認識されている。気候変動に関する政府間パネルは、2030 年までに世界の人為的な二酸化炭素排出量を 2010 年の排出量の約 45%を削減し、2050 年には「実質的なゼロ」を目標に設定することを決定しているが、これらの目標を達成するためには抜本的な対策が必要である。

選択肢の一つとして、電力生産効率を向上させ、輸送や産業におけるエネルギー使用料の削減である。もう一つの選択肢としては、水素のような代替エネルギー源の開発であ。現在は、水素の生産は主に天然ガスの水蒸気改質を通じて行われている。再生可能資源からの水素生産へシフトすることで、化石燃料に対する世界の依存度を和らげるのに役立つと考えられる。有望な選択肢の 1 つに、リグノセルロース系バイオマスからの生物学的水素生産がある。

リグノセルロース系バイオマスからの高塩分耐性菌を利用したバイオ水素生産はコンタミを防ぐための滅菌処理と加水による希釈（主に塩分の阻害を軽減するため）のための水の使用量の削減に貢献できる可能性がある。リグノセルロース系バイオマスは、森林、農業廃棄物等の自然由来のものであり、かつ大量に利用可能であるが、セルロース、ヘミセルロース、リグニン等の難分解性の構造を有している。これらの内、セルロースおよびヘミセルロースについては、物理的な処理によるサイズ低減、前処理、さらに酵素糖化の適用後に発酵プロセスによって生物学的に利用可能である。NaOH によるアルカリ前処理は、リグノセルロース系バイオマスに対して最も広く使用され、費用対効果が高いと考えられている。高塩分耐性菌はを用いることで、発酵プロセスの前に必要となる NaOH によるリグノセルロース系バイオマスの前処理とその後の HCl による中和後の希釈（主に塩分の阻害を軽減するため）に必要な水の量を比較的抑えることができる。

高塩分耐性を持つ水素産生菌は、高塩分濃度条件下で生育できる能力を有することから研究されてきた。この特性に基づき、高塩分耐性を持つ水素産生菌が高濃度 Na+イオン耐性を有すると仮定した。この仮説を検証するために、タイ国のコンケンの塩害土壌とバンコク近郊（サムット・サコーン地区）の塩田の土壌から得られた高塩分耐性水素産生菌をスクリーニングするとともにその特徴を調べた。この予備研究の結果、馴致期間を 1 年とることによって、26%（6M）の NaCl 飽和濃度条件下での水素生産が可能であることが示された。

次に、バンコク近郊（サムット・サコーン地区）の塩田の土壌から得られた高塩分耐性水素生産菌のグルコースからのバイオ水素生産に対する最適条件を

把握するための実験を実施した。結果から最適条件（26%NaCl、35°C、および pH9）下において 1.45 mol-H2/mol-グルコースの水素収率が得られた。またこの馴致した高塩分耐性水素生産菌は、様々な種類のリグノセルロース由来の糖および D-フルクトースを分解することができ、食品廃棄物からバイオ水素を生成する潜在的な能力を持つことが示唆された。分子生物学的な解析手法の 1 つである PCR-DGGE 法によって、混合培養系での優占菌は *Halanaerobium fermentans* であることが明らかとなった。

新しい 16s rRNA 配列の *Halanaerobium fermentans* B4 株が同定され、GenBank データベースに提出した（受付番号 MN133965）。この 1,424 ベースペアの塩基配列は、日本で塩漬けにされた魚の卵巣から分離され、2000 年に報告された *Halanaerobium fermentans* R-9 株と 99.36%の類似性を有していた。これらの知見は、バイオ水素生産のための高塩分耐性水素生産菌の適用に関する可能性を示すものと考えられる。

タイの塩田と塩害土壌から得られたサンプル 3 つを培養した高塩分耐性水素生産菌に関する微生物群集に関する研究を実施した。この培養は 26%NaCl 濃度条件下で増殖し得るバイオ水素生産細菌をスクリーニングすることを目的とした。16s rRNA の V3 領域におけるポリメラーゼ連鎖反応ー変性剤濃度勾配ゲル電気泳動（PCR-DGGE）法の結果と 16s rRNA の V3-V4 領域におけるハイスループットアンプリコンシーケンシングを IonS5™XL プラットフォーム技術によって行った結果とを比較した。

いくつかのバンドは、DGGE の結果から *Halanaerobium* と同じ属と一致し、高塩分耐性水素生産菌群のおおよそのプロファイルを提供できた。ハイスループットアンプリコンシーケンシングの結果から、すべての菌叢の中で最も高いポピュレーション：OTU(37～98%)は *Halanaerobium fermentans* R-9 株との密接な類似性を示したが、1 つの高いポピュレーション OTUs(61%)を示した菌叢は *Halanaerobacter lacuranum* TB21 と高い類似性を示した。 高いポピュレーションを有する *Halanaerobium fermentans* により高い水素モル収率（1.15 および 1.08 mol-H2/mol-グルコース）が得られ、一方で *Halanaerobacter lacuranum* は比較的高い値が得られた（0.66 mol-H2/mol-グルコース）。以上のように、次世代シーケンサ（NGS）により、PCR-DGGE 技術と比較して高塩分耐性水素生産菌群のより詳細でかつ定量的な情報が得られた。

ACKNOWLEDGEMENT

I would like to extend my sincere and deepest gratitude to my supervisor, Professor Tsuyoshi Imai, for his kindness, guidance, and support. I have learned so many things during the time I studied under his supervision.

My deepest appreciation to my graduate committee, Professor Masahiko Sekine, Professor Masakazu Niinae, Professor Takaya Higuchi, and Professor Eiichi Toorisaka, who have helped me so much to improve the quality of my work with their constructive suggestions.

Associate Professor Koichi Yamamoto and Associate Professor Ariyo Kanno have helped to shape my critical thinking during the Environmental Symbiosis Seminar in the EISEI laboratory. To them, I offer a sincere gratitude.

I also express a sincere thanks to Associate Professor Sompong O-Thong, Professor Alissara Reungsang, Associate Professor Prapaipid Chairattanamanokorn, and Dr. Siriporn Lunprom, for their guidance and suggestions.

I gratefully acknowledge The Directorate General of Higher Education (DIKTI) and the Indonesian Endowment Fund for Education (LPDP) for providing BUDI-LN scholarship.

I owed so much to Miss Toshimi Yamamoto, who has patiently helped me in the preparation of experiments.

I acknowledge Kyoko Ikeda and Hana Watari for establishing the pathway in the long study of these halophiles. I also thank Yoshiki Tsuda for his valuable contribution in data collection.

I'm not lonely in this doctoral endeavor with the support and friendship of the members of the EISEI lab: Nguyen Kim Diem Mai, Shahira Aly, Gde Yudha Partama, Natha Okajima, Uyen Doan To, Wang Jianing, Sativandi Riza, Passaworn Warunyuwong, Rafitah Hasanah, Jittrera Buates, Atin Nuryadin, Masato Fukushima, Kanathip Promnuan, Riku Motohashi, Hideya Saeki, Enamul Kabir, Ganjar Samudro, and all the master and B4 students. Thank you all very much.

To all my colleagues in Institut Teknologi Nasional Bandung, especially at the Environmental Engineering Department. I hope I could contribute more with the new knowledge I've learned in this study.

I heartily acknowledge my beloved husband who has given his fullest support during my study, my children, my mother, my siblings, and my grandmother for their encouragement. I dedicate this dissertation to my late father who had always believed in me.

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LIST OF ABRREVIATIONS

µmol	micromolar
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search tool
bp	base pairs
$CaCl_2 \cdot 2H_2O$	calcium chloride dihydrate
$CH_3(CH_2)_2COOH$	butyric acid
CH_3COOH	acetic acid
Cl^-	chloride ion
CO_2	carbon dioxide
$CoCl_2 \cdot 6H_2O$	cobalt(II) chloride hexahydrate
d	day
DBF_t	dry biogas factor (L dry at 0°C (L measured at *C*)$^{-1}$)
ddTTP	2',3'-dideoxythymidine triphosphate
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
e.g.	*exempli gratia* (for example)
EtBr	ethidium bromide
F/M	food to microorganism
$FeCl_3 \cdot 6H_2O$	iron(III) chloride hexahydrate
g	gram
g/L	gram/liter
Gb	Gigabyte
GC	guanine cytosine
GYP	glucose yeast peptone
h	hours
H_2	hydrogen
HAc	acetic acid

HBu	butyric acid
HHV	higher heating values
HMY	Hydrogen Molar Yield
IPCC	Intergovernmental Panel on Climate Change
K^+	potassium ion
K_2HPO_4	dipotassium phosphate
kb	kilobyte
kbp	kilo base pairs
KCl	potassium chloride
L	liter
LHV	lower heating values
M	molar
m	meter
Mb	Megabyte
$MgCl_2 \cdot 6H_2O$	magnesium chloride hexahydrate
$MgSO_4 \cdot 7H_2O$	magnesium sulfate heptahydrate
min	minutes
mL	milliliter
mM	millimolar
MUSCLE	multiple sequence comparison by log-expectation
Na^+	sodium ion
Na_2SO_4	sodium sulfate
NaCl	sodium chloride
NADH	reduced nicotinamide adenine dinucleotide
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
NGS	next generation sequencing
NH_4Cl	ammonium chloride

$(NH_4)_2HPO_4$	diammonium phoshate
NI	no information
$NiCl_2 \cdot 6H_2O$	nickel(II)chloride hexahydrate
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PD	phylogenetic diversity
PHA	polyhydroxybutyrate
R/P	reserves-to-product
rRNA	ribosomal ribonucleid acid
SSU	small subunit
STP	standard temperature and pressure (0°C, 1 atm)
T	temperature
TAE	tris-acetate-EDTA
Taq	*Thermophilus aquaticus*
Tb	Terabyte
v/v	volume/volume
V_0	dry (non-water) biogas volume at 0°C (L)
VFA	Volatile Fatty Acid
V_t	biogas volume measured at temperature *T* (L)
w/w	weight/weight
WGS	water gas shift
WMO	World Meteorological Organization
wt./vol.	weight/volume

CHAPTER I

INTRODUCTION

1.1 Rationale and background

The growing energy demand in the world has created a strong dependence on fossil fuels. The burning of fossil fuels, which produces carbon dioxide, has led to global warming and climate change. Since fossil fuels are unrenewable, this dependence, which has led to depletion of the resources along with the alarming rate of global warming, has initiated many kinds of research toward alternative energies. Hydrogen is among those renewable and clean energies.

As the United States led the hydrogen fuel initiatives in 2003, several countries and states in the world such as Japan, Mexico, and Taiwan, have taken the measures to shift to hydrogen economy and society (Lee and Lee, 2008; Behling, Williams and Managi, 2015; López Ortiz, Meléndez Zaragoza and Collins-Martínez, 2016). Although hydrogen is an efficient and clean energy carrier, only 4% of the world's total hydrogen production comes from renewable sources (Parthasarathy and Narayanan, 2014). The other 96% still comes from fossil fuel sources, such as coal gasification and natural gas reforming. Hydrogen production from renewable sources is possible through several biological pathways, including dark fermentation which has been considered as a practically feasible option (Łukajtis *et al.*, 2018).

Dark fermentation offers high yields per mol of substrate, has no oxygen limitation due to the fully anaerobic process and a great variety of carbon sources available as substrates (Dipasquale *et al.*, 2014; Boboescu *et al.*, 2016). As a part of the second-generation feedstocks for biofuels, lignocellulosic biomass has several advantages compared to the first-generation feedstocks which come from food crops. It does not compete with food production and highly available since the annual production of lignocellulosic biomass residue of the world is estimated to reach more than 220 billion tons (Kumar *et al.*, 2015). But the cellulose, hemicellulose, and lignin composition of the lignocellulosic biomass have made

it naturally recalcitrant. Pretreatment and enzymatic saccharification of hemicellulose and cellulose are required before bacterial fermentation can take place to produce fuels (Kim, Lee and Kim, 2016). One of the most cost effective and widely used pretreatment process for lignocellulosic biomass is alkaline pretreatment with NaOH (Kim, Lee and Kim, 2016).

After pretreatment with NaOH, the lignocellulosic biomass need to be neutralized by dilution with water to continue to the next step of the process, which is enzymatic saccharification. In a large scale of operation, the quantity of water needed for the neutralization will translate into a higher cost. This is where halophilic bacteria can play a part. Halophilic bacteria require NaCl for growth. The extreme halophiles have the most rapid growth at 20 to 30% of NaCl (Ollivier *et al.*, 1994). By utilizing halophilic bacteria for dark fermentation of the pretreated and saccharified lignocellulosic biomass, the neutralization process can be done with the addition of lower quantity of HCl instead of higher quantity of water dilution. The near saturation condition at 26% NaCl where the bacteria was acclimatized may be beneficial for application in solid-state fermentation process.

Another advantage of biohydrogen production by extremely halophilic bacteria is that the high salt concentration naturally inhibits the methanogens. Methanogenesis and sulfate reduction from hydrogen and acetate produced by fermentation has been reported to be very limited in NaCl concentration higher than 15% (Oren, 1988). This advantage can contribute to eliminating possible complications caused by methane and hydrogen sulfide; thus, better hydrogen recovery and purification by membranes can be achieved.

1.2 Dissertation objectives

This study aims to investigate hydrogen production by extremely halophilic mixed culture enriched from a salt pan and salt damaged soil through dark fermentation. Five sub-objectives have been formulated to obtain the goal mentioned above:

1. To evaluate the hydrogen production under different salinity concentrations before and after acclimatization period of 1 and 2 years.

2. To find the optimum condition (pH, temperature, and salinity) for biohydrogen production for one of the bacterial communities.
3. To identify the predominant hydrogen producers among the acclimatized mixed culture.
4. To examine the ability of the bacteria to ferment various types of carbohydrates.
5. To analyze the bacterial communities through PCR-DGGE and Next Generation Sequencing.

1.3 Structure of the dissertation

This dissertation is divided into seven chapters and listed as follows:

- **Chapter I** states the rationale and background, objectives, and structure of the dissertation.
- **Chapter II** explores the extensive background of the theories, methods, and summary of previous studies.
- **Chapter III** evaluate the hydrogen production under different salinity concentrations before and after acclimatization period of 1 and 2 years.
- **Chapter IV** examines the optimum condition for biohydrogen production for one of the bacterial communities.
- **Chapter V** discusses the identification of the predominant hydrogen producers and examines the ability of the bacteria to ferment various types of carbohydrates.
- **Chapter VI** recapitulates the results of the study and offers suggestions for future studies.

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CHAPTER II

LITERATURE REVIEWS

2.1. The current state of the world's energy and global warming

In the twenty first century, the world saw the highest population growth and a better standard of living that prompted an increase global energy demand (Dincer and Acar, 2014). As much as 81% this energy demand in 2018 was still fulfilled from fossil fuel sources of oil, coal, and natural gas (Figure 2.1).

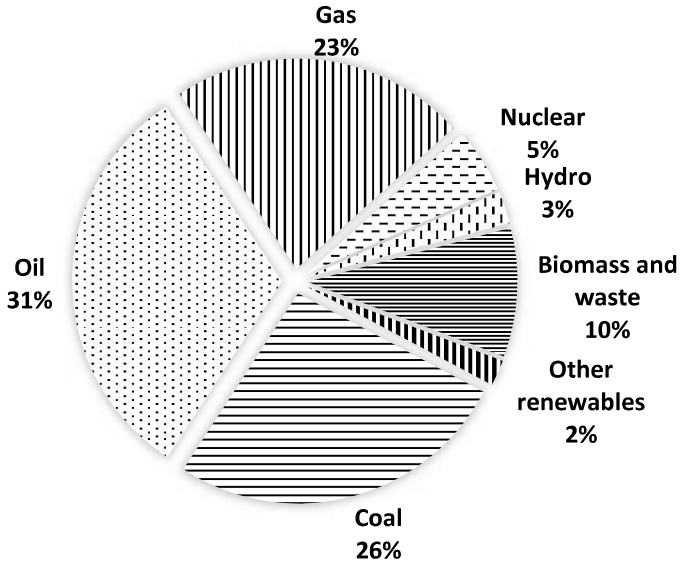


Figure 2.1 – Global energy demand by the source in 2018 (International Energy Agency, 2018)

As an unrenewable resource, global fossil fuels rely on production from oil, coal, and gas reserves discovered through explorations. The reserves-to-product (R/P) ratio measures the number of years of current production of global oil, natural gas, and oil based on known reserves and annual production. From the R/P ratio, the number of years of these resources' availability can be predicted (Figure 2.2).

With only 50, 50.9, and 132 years left for the world oil, natural gas, and coal reserves, the world needs to wean the dependency on fossil fuel. Carbon dioxide emitted by the combustion of fossil fuel has contributed to global warming. The record has shown that the world average temperature between 2015 to 2018 was the warmest on the record (WMO - World Meteorological Organization, 2019). The Intergovernmental Panel on Climate Change is aiming to lower the global net human-caused emissions of carbon dioxide by about 45% by 2030 from the 2010 levels and set the goal of “net zero” in 2050 (WMO - World Meteorological Organization, 2019).

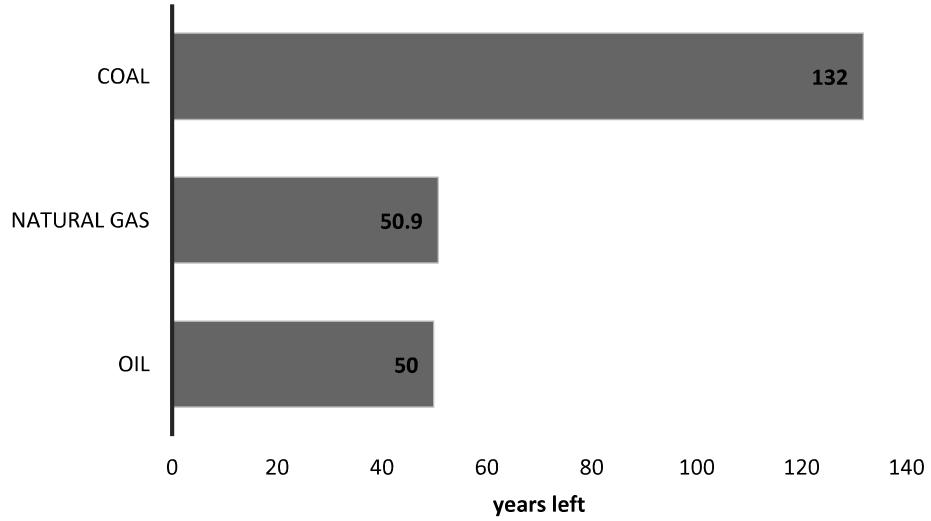


Figure 2.2 – Years of global fossil fuel reserves left, reported as the reserves-to-product (R/P) ratio in 2018. The values were based on the current production and can change with time upon the annual production and discovery of new reserves (BP, 2019)

The Unites Nations’ 2030 Agenda for Sustainable Development has the Sustainable Development Goals at its core, including Goal 7, affordable and clean energy. Goal 7.2 aims to increase substantially the share of renewable energy in the global energy mix. Countries are encouraged to accelerate the transition for sustainable energy system through investing in renewable energy

resources and adopting clean energy infrastructure and technologies to reach Goal 7.b (United Nations Division for Sustainable Development, 2017).

2.2. Hydrogen as an energy carrier

Among the alternative energies, hydrogen has several advantages, which are: near-zero or zero-end use emissions, high efficiency of energy conversion, having the highest energy density, high heating values, and lower heating values among other fuel sources (Table 2.1 and 2.2), and has different forms of storage as liquid, gaseous or together with metal hydrides (Dincer and Acar, 2014).

However, 96% of the world's total hydrogen still relies on fossil fuel sources and only 4% comes from other alternative sources (Figure 2.3). Hydrogen production methods are also not maturely developed yet, which contributes to low efficiencies and high production costs (Acar and Dincer, 2014).

2.3. Hydrogen production through biological pathways

Hydrogen production through biological pathways is a promising way to produce hydrogen from renewable sources. Although currently biohydrogen only contributed a small part to global production, the part is expected to grow with ongoing researches to improve the systems and techniques. Compared to physical and chemical processes, biological hydrogen production mostly has the advantage of operating at ambient pressure and temperature, thus require less energy and fewer costs. The versatility of carbon sources for biological hydrogen production made the process can be applied to waste material as feedstocks (Abdalla *et al.*, 2018). Living microorganisms can regenerate to produce newly replicated cells, contributing to a longer lifetime compared to inorganic catalysts, which can be fouled during operations (Alfano and Cavazza, 2018).

The main mechanism of biohydrogen production can be classified into photobiological production, biological water gas shift (WGS) reaction, and dark fermentation. Photobiological production is further divided into direct biophotolysis, indirect biophotolysis, and photofermentation. In direct biophotolysis, Fe-hydrogenase enzymes of green algae chloroplasts such as

Chlorella fusca, Chlamydomonas renhardtii, and *Secenedesmus obliquus* catalyze the split of water molecules into oxygen, protons, and electrons as shown in Equation 2.1 (Drapcho, Nhuan and Walker, 2008).

Table 2.1 – Energy density values at STP for common fuels (Drapcho, Nhuan and Walker, 2008)

Fuel source	Energy density (kJ/g)	Density (kg/m^3)	Energy content (GJ/m^3)
Hydrogen	143.0	0.0898	0.0128
Methane (natural gas)	54.0	0.7167	0.0387
No. 2 diesel	46.0	850	39.1
Gasoline	44.0	740	32.6
Soybean oil	42.0	914	38.3
Soybean biodiesel	40.2	885	35.6
Coal	35.0	800	28.0
Ethanol	29.6	794	23.5
Methanol	22.3	790	17.6
Softwood	20.4	270	5.5
Hardwood	18.4	380	7.0
Rapeseed oil	18.0	912	16.4
Bagasse	17.5	160	2.8
Rice hulls	16.2	130	2.1
Pyrolysis oil	8.3	1280	10.6

$$2H_2O \xrightarrow{light\ energy} O_2 + 4H^+4e^- \quad (2.1)$$

The mechanism in indirect biophotolysis involves two steps. During the first step, the light energy is used to produce stored carbohydrate and oxygen. The forms of carbohydrate are usually glycogen in cyanobacteria like *Spirulina* and *Anabaena variabilis* and starch in green algae. These carbohydrates are converted to hydrogen and carbon dioxide in a light driven process of the second step of indirect biophotolysis under oxygen-depleted conditions following Equation 2.2 and 2.3 (Drapcho, Nhuan and Walker, 2008).

$$6CO_2 + 12H_2O \xrightarrow{light\ energy} C_6H_{12}O_6 + CO_2 \quad (2.2)$$

$$C_6H_{12}O_6 + 6H_2O \xrightarrow{light\ energy} 12H_2 + 6CO_2 \quad (2.3)$$

Purple non-sulfur photosynthetic bacteria such as *Rhodopseudomonas sphaeroides* or *R. capsulate* have the ability to produce biohydrogen with their nitrogenase enzyme in the photofermentation process. The process requires nitrogen limitation under anaerobic conditions. The light stimulated the nitrogenase's hydrogen producing activity with adequate energy input (Equation 2.4), but the process is strongly inhibited by ammonium, oxygen and nitrogen gas. Although hydrogenase enzymes have an important role in direct photolysis, the enzyme consumes hydrogen in photofermentation process (Drapcho, Nhuan and Walker, 2008).

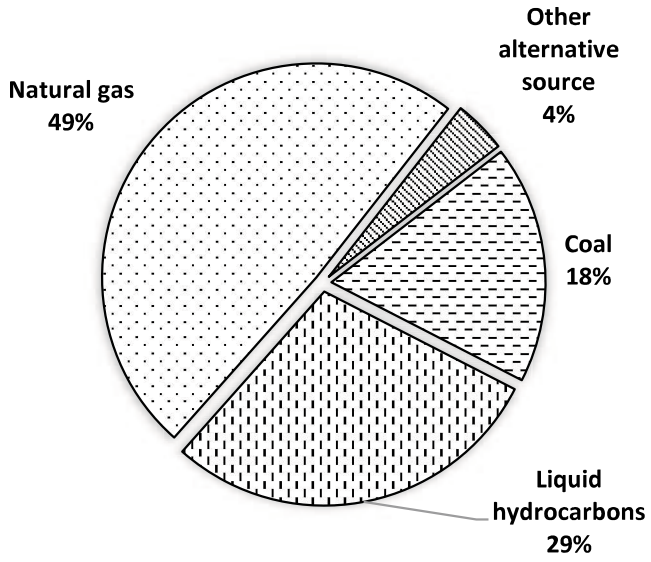


Figure 2.3 – Hydrogen production from various sources (Parthasarathy and Narayanan, 2014)

$$2H^+ + 2e^- + 4ATP \xrightarrow{light\ energy} H_2 + 4ADP + 4P_1 \quad (2.4)$$

While photobiological hydrogen production has great potential because of the immense energy available in solar radiation, it also has the limiting factor of low conversion efficiency of solar energy. Around 90% of the captured solar radiation

is released primarily as heat instead of utilized in photosynthesis (Drapcho, Nhuan and Walker, 2008).

Table 2.2 – Higher heating values (HHV) and lower heating values (LHV) of hydrogen and common fossil fuels at 25°C and 1 atm (Dincer and Acar, 2014)

Fuel source	HHV (kJ/g)	LHV (kJ/g)
Hydrogen	141.9	119.9
Methane	55.5	50.0
Gasoline	47.5	44.5
Diesel	44.8	42.5
Methanol	20.0	18.1

Biological water gas shift employs photoheterotrophic bacteria such as *Rhodospirillum rubrum* (Zürrer and Bachofen, 1979) and *Citrobacter amalonaticus* (Ainala *et al.*, 2017) to utilize carbon monoxide as their sole carbon source in the dark. The ATP is generated by coupling the carbon monoxide's oxidation to the reduction of H^+ to H_2, as shown in Equation 2.5 (Abdalla *et al.*, 2018).

$$CO + H_2O \rightarrow CO_2 + H_2 \quad (2.5)$$

The conversion in biological WGS employs [NiFe]-CO dehydrogenase to oxidize carbon monoxide to carbon dioxide and [NiFe]-hydrogenase which produces hydrogen. The limiting factor of WGS includes carbon monoxide toxicity towards bacteria due to the high affinity of carbon monoxide for metalloenzymes, which inhibit the bacterial growth and catabolic activity(Alfano and Cavazza, 2018).

2.3.1. Biohydrogen production through dark fermentation

In biohydrogen production through dark fermentation, chemoorganotrophic microorganisms use organic substrates as their energy as well as carbon sources and H^+ as their electron acceptor under anaerobic conditions. Along with

hydrogen production, volatile fatty acids such as acetate, butyrate, lactate, or propionate are produced, while alcohols such as ethanol are produced in a smaller amount. The rates of formation and quantity of these products differ between organisms, while substrate and culture conditions such as temperature, pH, and dissolved hydrogen concentration also play part.

Theoretically, microbial fermentation follows equation 2.6 for acetic acid (HAc) formation and equation 2.7 for butyric acid (HBu) formation:

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2 \quad (2.6)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3(CH_2)_2COO^- + 2HCO_3^- + 3H^+ + 2H_2 \quad (2.7)$$

Following equation (1), 4 mol hydrogen will be produced from 1 mol glucose. Hence, 498 mL of hydrogen will be produced from 1 gram of glucose at standard temperature and pressure (STP) conditions through the HAc pathway. For the HBu pathway in Equation (2.7), 2 mol hydrogen will be produced from 1 mol glucose. Therefore, 249 mL H_2 will be produced from 1 gram of glucose through the HBu pathway at STP conditions.

The efficiency of the fermentative process is compared with the hydrogen molar yield (HMY), which is the value of cumulative hydrogen produced (mol H_2) divided by glucose provided in the substrate (mol glucose). Theoretically, the maximum HMY for glucose in anaerobic fermentation is 4, which can be reached through the HAc pathway (Equation 1).

The dark fermentation process offers several advantages in industrial biohydrogen production. Among them are high production rates, high yields per mole of substrate, continuous production regardless of solar light condition (Dipasquale *et al.*, 2014), high variety of carbon sources as substrates, and has no oxygen limitation since the process is fully anaerobic (Hallenbeck, Abo-Hashesh and Ghosh, 2012; Boboescu *et al.*, 2016). On the other hand, dark fermentation also has several limitations, such as thermodynamically unfavorable conditions as hydrogen yields increases and carbon dioxide's presence in the produced gas (Boboescu *et al.*, 2016).

2.4. Lignocellulosic biomass

Lignocellulosic biomass is a term for the plant biomass derived from grasses, softwood, hardwood, and agricultural wastes (Hassan, Williams and Jaiswal, 2018). Among carbon sources to supply fermentable sugars in biohydrogen production, lignocellulosic biomass is a highly considered option. It doesn't compete with food production, available abundantly in nature as grasses and woods, in forestry and agricultural residues, as well as in domestic and industrial solid wastes. It was estimated that lignocellulosic biomass residue is being produced more than 220 billion tons annually all over the world (Kumar *et al.*, 2015).

However, the structure of lignocellulosic biomass, which consists of cellulose, hemicellulose, and lignin, is naturally recalcitrant. Hemicellulose and cellulose, the polysaccharides part of lignocellulosic biomass, can be biologically utilized after the application of several stages of unit processes, as follows (Kim, Lee and Kim, 2016): size reduction through mechanical means, pretreatment to make cellulose more readily available for enzyme conversion, enzymatic saccharification of cellulose and hemicellulose to hydrolyze into fermentable sugars, microbial fermentation involving conversion of fermentable sugars into fuels by bacteria, and product recovery and purification. Among those processes, pretreatment has been considered as one of the most costly (Den *et al.*, 2018), although alkaline pretreatment with NaOH is considered as the most widely used and cost-effective for lignocellulosic biomass (Kim, Lee and Kim, 2016).

Cereal residues such as sugarcane bagasse, corn stover, rice straw, and wheat straw, has a high fraction of lignocellulosic substances, making them the chosen feedstock for many biorefineries (Hassan, Williams and Jaiswal, 2018). Lignocellulosic-derived sugars include glucose, galactose, mannose, xylose, arabinose, fucose, and rhamnose (Mosier *et al.*, 2005; Jönsson and Martín, 2016). Table 2.3 shows the composition of several lignocellulosic biomass retrieved from the Netherlands Energy Research Foundation (ECN) Phyllis2 database (ECN, 2013) and other sources.

Table 2.3 – Cellulose, hemicellulose, and lignin of lignocellulosic feedstocks (% dry basis)

Source	Cellulose	Hemicellulose	Lignin	References
Cereal residues				
Sugarcane bagasse	42.40	35.30	20.80	(Garcìa-Pèrez, Chaala and Roy, 2002)
Corn stover	36.30	25.40	16.90	(Evans *et al.*, 1988)
Rice straw	32.10	24.00	12.50	(Richard and Trautmann, 1996)
Wheat straw	44.50	30.10	8.00	(Singh *et al.*, 1996)
Grasses				
Hemp	53.6	10.60	8.76	(Raud *et al.*, 2016)
Bamboo	40.25	32.01	24.45	(Khan *et al.*, 2018)
Reed	49.60	31.50	8.74	(Antal Michael Jerry *et al.*, 2000)
Softwood				
Japanese cedar	52.7	13.8	33.5	(Muranaka *et al.*, 2017)
Pine	45.6	24	26.8	(Yu *et al.*, 2017)
Spruce	47.1	22.3	29.2	(Yu *et al.*, 2017)
Hardwood				
Rubber wood	39.56	28.42	27.58	(Khan *et al.*, 2018)
Oak	43.2	21.9	35.4	(Yu *et al.*, 2017)
Eucalyptus	44.9	28.9	26.2	(Muranaka *et al.*, 2017)

2.5. Microbial community analysis

In the 1870's, Robert Koch and Julius Petri have isolated bacteria for scientific purposes for the first time. It was evident to Koch that different bacteria require different settings for optimal condition, and it was generally accepted by the early 1900s that the majority of bacteria couldn't be cultivated with standard techniques

(Hugerth and Andersson, 2017). This phenomenon can happen due to several reasons. The bacteria might be dependent on metabolites produced by their community members, or oxidative stress caused by laboratory settings. Some bacteria may grow at a slow pace to be recognized in the laboratory (Hugerth and Andersson, 2017).

Direct microscopy came as an alternative to bacterial culturing. However, some difficulties occur in obtaining taxonomic resolution for the diverse microbes present in the environmental sample. Molecular fingerprinting has its roots in 1977 when the small subunit (SSU) of the ribosomal RNA (rRNA) was established to be a suitable gene for inferring phylogenetic relationships of organisms by Woese and his colleagues. This technique was soon applied to natural communities by Norman Pace and his colleagues in 1985 (Hugerth and Andersson, 2017).

2.5.1. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a method for the amplification of ribosomal rRNA gene sequences. PCR was invented by Kary Mullis in 1983. After further developments by his colleagues at Cetus Corp and Henry Erlich's laboratory, the PCR method was gaining high popularity in the 1990s. One of the pivotal breakthroughs in PCR method development was the isolation of thermostable polymerase enzyme from *Thermophilus aquaticus* (*Taq*), which was isolated from thermal springs (Bartlett and Stirling, 2003). The thermostable enzyme enables the denaturation at 95°C as the first step of PCR, where the two strands of the DNA double helix are separated by breaking the hydrogen bonds. The second step of PCR is annealing of the primers to each DNA strand at 45 to 55°C. The final step is the extension step, where the *Taq* polymerase enzyme synthesizes new DNA strands, which are complementary to the DNA template strand, from the free nucleotides (dNTPs). The enzyme functions optimally at 72°C and gives a high fidelity, eliminating the formation of nonspecific products due to less stringent primer binding. These three steps are usually repeated for 20 to 30 times in a thermocycler.

PCR starts with the extraction of nucleic acid templates. Several popular DNA extraction methods from environmental samples include freeze-thaw and detergent lysis procedures which were introduced in 1997 and 1991 (LaMontagne *et al.*, 2002). Application of bead mill homogenization can effectively extract DNA at the cost of DNA template shearing. Freeze-thaw procedures and hot-detergent lysis methods can avoid this DNA shearing, but co-extract humic acid can inhibit the enzymes for DNA extraction, thus gave a lower yield (LaMontagne *et al.*, 2002).

2.5.2. Denaturing gradient gel electrophoresis (DGGE)

Denaturing gradient gel electrophoresis was introduced by Gerard Muyzer in 1993. The method is based on the electrophoresis of the 16S rDNA fragments, which have been amplified by PCR, in a linearly increasing gradient of denaturants in polyacrylamide gels (Muyzer, de Waal and Uitterlinden, 1993). DGGE employs a primer with an additional 40-nucleotide GC-rich sequence (GC-clamp) at the 5' end that provided stability of transitional molecules (Muyzer, de Waal and Uitterlinden, 1993) , so that the fragment will stop at the position of denaturant concentration that corresponds to the GC (guanine and cytosine) content of the fragment. After electrophoresis, the polyacrylamide gels are stained with DNA binding fluorescent dyes such as ethidium bromide (EtBr) or SYBR Green so they can be visualized under the ultraviolet light.

The relative abundance of the microorganisms can be estimated through their band's intensity compared to the other band's intensities in the corresponding sample. The number of bands in each lane can show a comparison of the relative abundance between communities. For identification of the microbial community members, important bands from the sample lanes of the gel can be excised, reamplified, cleaned, and send for sequencing. Although PCR-DGGE offers the advantage of immediate display of the genetic diversity of microbial communities, it also has several limitations, which are: (a) it can only separate relatively short (~ 500 bp) DNA fragments (Muyzer, 1999), and (b) only major constituents in community could be identified (Kumar *et al.*, 2018).

2.5.3. Sanger sequencing

DNA sequencing techniques were first developed by Maxam & Gilbert and Sanger & Coulson. In 1977, Sanger and Coulson published the papers on the rapid determination of DNA sequence, which provided the distinctive advantage of reduced handling of radioisotopes and toxic chemicals (Schuster, 2008). The method employs the dideoxy terminator chemistry. The dideoxynucleotides, or also known as 2',3'-dideoxythymidine triphosphate (ddTTP), act as inhibitors that terminate the newly synthesized chains at specific residues (Sanger, Nicklen and Coulson, 1977). The automated Sanger sequencing method, which is used today has been combined with the usage of fluorescent dyes, which are labeled at the 5' end of a primer, which was developed by Leroy Hood and his colleagues. The fluorescent dyes emit light at different wavelengths, which was detected by capillary electrophoresis. Although this method is considered as the gold standard for sequencing, Sanger sequencing has several limitations, which are (Fakruddin and Chowdhury, 2012): (a) the need for polymers or gels as sieving media for fluorescent-labeled DNA fragments, (b) difficulty in total automation of the sample preparation, (c) high cost of sequencing, and (d) only a relatively low number of samples can be analyzed in parallel.

2.5.4. Next generation sequencing (NGS)

Next generation sequencing (NGS) or high-throughput sequencing are catchall terms for non-Sanger-based sequencing technologies. The development of NGS was driven by the human genome project, which was launched in 1990 and completed in 2003. The first sequencing-by-synthesis technology was launched in 2005, which was developed by 454 Life Sciences and George Church's lab's multiplex polony sequencing protocol. This sequencing-by-synthesis technology highly extended the number of sequencing reactions while greatly reduces the required reaction volumes (Schuster, 2008). The current sequencing platforms can read between 1 kb to 2 Mb, with throughput range from 300 kb to 5.2 Tb and run time between 1 minute to 3.5 hours (Table 2.4). NGS has several advantages compared to Sanger sequencing, which are: (a) it can

analyze millions of templates parallelly at a time, (b) it can provide quantitative analysis of bacterial communities instead of qualitative, (c) it can provide higher coverage, and (d) it can identify minor constituents of the microbial communities.

2.6. Halophilic bacteria

In recent years, many research efforts have been made to replace the petroleum based chemical industry with industrial biotechnology. One of the challenges that face bio-based products such as biofuels, bioplastics, and biochemicals, is that their production costs are still too expensive to compete with the chemical-based products. Several issues have been associated with the high production cost of bioprocessing, among them were (Yin *et al.*, 2015): (a) Bioprocessing consumes large amount of fresh water, (b) the price of pure substrates, such as glucose which are produced by hydrolysis from starch, have shown a high increase over time, (c) the sterilization of medium, fermenters, and piping systems is energy intensive, (d) to avoid contamination, most of fermentation process have to be run discontinuously, which contributes to low efficiency, and (e) complicated procedures for to ensure the batch processes are free from contaminants contributes to the high cost of biochemical production.

The ability of halophilic bacteria to contribute in less consumption for fresh water and energy and also to reduce process complexity for industrial application has been shown in the production of polyhydroxybutyrate (PHA) by *Halomonas* TD01 and *Pseudomonas entomophila* (Tan *et al.*, 2011; Wang, Yin and Chen, 2014). The bacteria have shown the ability to grow in seawater in a continuous and unsterile way without any contaminations and good subjects of genetic manipulation for the construction of super PHA production strains (Fu *et al.*, 2014).

Halophilic and halotolerant bacteria are present in a wide range of habitats such as hypersaline soils, saltern ponds, saline lakes, desert, and salted foods. Halophilic bacteria have been first mentioned in the work of Hof, who inoculated salt mud from a solar saltern in Java in to media with various salinities in 1935 (Ventosa, Nieto and Oren, 1998). She isolated an endospore-containing *Bacillus* species, which was able to grow at 24% NaCl and *Pseudomonas beijerinckii* from

salted beans preserved in brines in 12 and 18% salt media. However, during early research on the hypersaline environment, the microbiology study of halophilic bacteria was often eclipsed by the red halophilic archaea (Ventosa, Nieto and Oren, 1998).

Table 2.4 – Sequencing platforms available in 2019 (adapted from Besser *et al.*, 2018)

Platform/ Instrument type	Through-put range (Gb)	Read length (bp)	Run Time	Strength	Weakness
Sanger sequencing					
ABI 3500/3730	0.0003	Up to 1 kb	30-140 min	Read accuracy and length	Cost and throughput
Illumina					
iSeq 100	0.144-1.2	1×36 to 2×150	9-17.5 h	Low initial investment	Run and read length
MiniSeq	1.7-7.5	1×75 to 2×150	4-24 h	Low initial investment	Run and read length
MiSeq	0.3-15	1×36 to 2×300	4-55 h	Read length, scalability	Run length
NextSeq	10-120	1×75 to 2×150	12-30 h	Throughput	Run and read length
HiSeq (4000)	10-1500	2×150	<1-3.5 d	Read accuracy, throughput	High initial investment, run
NovaSeq 6000	2000-6000	2×50 to 2×250	13-44h	Read accuracy, throughput	High initial investment, run
Ion Torrent					
PGM 318	0.08-2	Up to 400	4-7 h	Read length, speed	Throughput, homopolymers[b]
S5 & S5 XL	0.3-15	Up to 600	2.5-4 h	Read length, speed	Homopolymers[b]

Platform/ Instrument type	Through-put range (Gb)	Read length (bp)	Run Time	Strength	Weakness
Proton	10-15	Up to 200	2-4 h	Speed, throughput	Homopolymers[b]
Pacific BioSciences					
Sequel	5-10[a]	Up to 60 kb	≤ 20 h	Read length, speed	High error rate
Sequel II	Up to 450	Up to 175 kb	10-30 h	Read accuracy and length	Run length
Oxford Nanopore					
MInION Mk1B & C	0.1-50	> 2Mb	1 min-48 h	Read length, portability	High error rate, run length
GridION Mk1	Up to 250	> 2Mb	1 min-48 h	Read length	Run length
PromethION 24 & 48	Up to 5200	> 2Mb	1 min-72 h	Read length, ultra-high throughput	Run length, high initial investment

[a] per one single-molecule real-time cell

[b] results in an increased proportion of reads containing errors among all reads which in turn results in false-positive variant calling

Halophilic bacteria differ from halotolerant bacteria in terms that they require NaCl for growth. Halophiles are classified by their response to NaCl, with slight halophiles has the most rapid growth at 2 to 20% (0.34-0.85 M) NaCl, moderate halophiles at 5 to 20% (0.85-3.4 M) NaCl, and extreme halophiles at 20 to 30% (3.4-5.1 M) NaCl (Larsen, 1962; Ollivier *et al.*, 1994). There have been some studies showing that higher salt concentration results in diverse volatile fatty acids and higher than the usual accumulation of hydrogen (Ollivier *et al.*, 1994).

2.6.1. Biohydrogen production by halophilic bacteria

Most of the fermentative halophilic bacteria belong to the order Halanaerobiales (previously named as Haloanaerobiales) under the phylum of Firmicutes and the class of Clostridia. Halanaerobiales consists of two families, which are the Halobacteriodaceae and the Halanaerobiaceae (previously named as Halanaerobiaceae) (Kivistö and Karp, 2011). Beside the twenty five fermentative species currently places under Halanaerobiales, there are one species of obligate halophilic fermentative bacteria belongs the order Natranaerobiales, and another species belongs to the order Clostridiales (Kivistö and Karp, 2011). *Halanaerobium prevalens*, which was isolated from the sediments of the Great Salt Lake of Utah and characterized in 1983, was the first discovered halophilic fermentative bacterial species (Kivistö and Karp, 2011).

A majority of the halophilic fermentative bacteria are moderate halophiles, but a few of them are extreme halophiles, such as *Acetahalobium arabaticum*, *H. lacurosei*, and *H. tunisiense,* which grow optimally under 150-180, 200-220, and 200 g/L of salt (Kivistö and Karp, 2011). Other than salt concentration between 5 to 30%, halophilic fermentative bacteria generally require obligate anaerobic conditions and simple organic compounds such as sugars and amino acids. Almost all of them are Gram-negative, excluding *H. tunisiense* (Kivistö and Karp, 2011). Most of the members of the Halanaerobiales can utilize a wide range of carbohydrates. Their typical fermentation products including acetate, hydrogen, carbon dioxide, and ethanol. Some of the members produce butyrate, formate, lactate, valerate, propionate and 1,2 – propanediol (Kivistö and Karp, 2011).

One of the concerns in the biohydrogen production is the existence of methanogenic archaea that will reduce the hydrogen as fermentation product into methane. Several strategies have been taken to inhibit these methanogens in enriched mixed cultures from natural environments taken as inoculum for biohydrogen production. Those strategies include heat-shock treatment, operation at low pH, and operation at a short hydraulic retention time (Hawkes *et al.*, 2002). Extreme halophilic bacteria have a potential role to fill several gaps in biohydrogen production from lignocellulosic biomass. The first one is that the high salt concentration naturally inhibits the methanogens. In NaCl concentration

higher than 15%, methanogenesis and sulfate reduction from hydrogen and acetate produced by fermentation has been reported to be very limited (Oren, 1988). The second one is with their ability to grow in a high concentration of NaCl. After lignocellulosic pretreatment with NaOH and neutralization with HCl, only a relatively small amount of water will be needed to dilute the pretreated biomass before fermentation process. For these reasons, the application of extremely halophilic bacteria for biohydrogen production from lignocellulosic biomass can potentially contribute to cost reduction of sterilization and water. But to date, there were still limited number of studies about biohydrogen production by halophilic bacteria (Table 2.5).

2.6.2. Hydrogen production pathway of halophilic bacteria

Ferredoxin exchange pathway is the most common hydrogen-producing exchange pathway associated with pyruvate metabolism in strict anaerobes. A study of the genome of *H. hydrogeniformans* (M. B. Begemann *et al.*, 2012) by using a combination of 454 and Illumina sequencing methods revealed the identification of pathways of acetate production from pyruvate (Figure 2.4). While the genome of *H. hydrogeniformans* has been reported to lack ferredoxin hydrogenase enzyme (Roush *et al.*, 2013), four [FeFe]-hydrogenases have been identified in *H. saccharolyticum* subsp. *Saccharolyticum* strain DSM 6643^T (Kivistö *et al.*, 2013). Out of the four [FeFe]-hydrogenases, two were putative bifurcating hydrogenases which require both reduced NADH and ferredoxin. *H. saccharolyticum* was sequenced using Illumina paired-end sequencing and 454 technologies (Kivistö *et al.*, 2013).

2.7. Solid-state fermentation (SSF)

Solid-state fermentation (SSF) is defined as fermentation which involves solids in the absence, or near absence of free water (Pandey, 2003). The substrate itself should retain enough moisture to support metabolism and growth of micro-organism. The technique has been traditionally used since ancient time, such as in the production of *miso*, *soy sauce*, *shochu* and *sake* by using *koji* molds

(*Aspergillus oryzae, A. sojoe, A. kawachii, A. shoriusamii* and *A. awamori)* (Kitamoto, 2002).

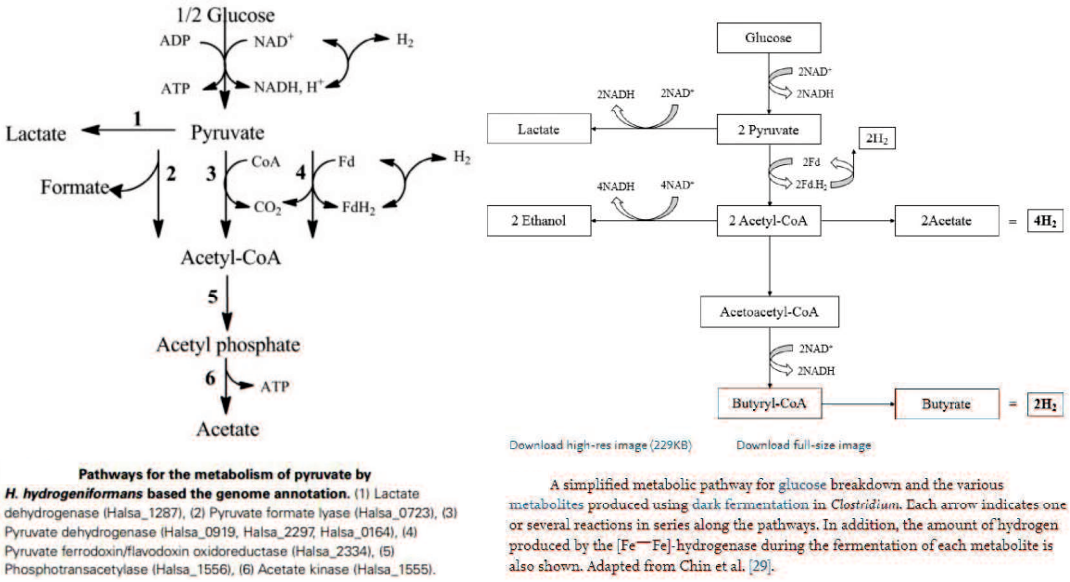
Table 2.5 – Comparison for hydrogen yield of fermentation by halophilic bacteria

Carbon source	Inoculum	NaCl	T (°C)	pH	HMY	Reference
Cellobiose	*Halanaerobium hydrogeniformans*	7%	33	11	2.3±0.2 mol H_2/ mol cellobiose	(M. Begemann *et al.*, 2012)
Glucose	*H. hydrogeniformans*	7%	33	11	2.42 mmol H_2/ mmol glucose	(M. Begemann *et al.*, 2012)
Glucose	*H. salinarius*	14-15%	NI	NI	2 mM/4.17 mM	(Mouné *et al.*, 1999)
Glucose	*H. chitinovorans*	12%	37	5	144.5 µmol H_2/ 5 mL 0.5% glucose	(Liaw and Mah, 1992)
Glycerol	*H. saccharolyticum senegalensis*	150 g/L	37	7	1.6 mol H_2/ mol glycerol	(Kivistö, Santala and Karp, 2010)
Glycerol	*H. saccharolyticum saccharolyticum*	150 g/L	37	7.4	0.62 mol H_2/ mol glycerol	(Kivistö, Santala and Karp, 2010)
Glucose	Mixed culture from salt factory wastewater	75 g/L	35	8	0.9 ± 0.02 mol H_2/mol glycerol	(Pierra *et al.*, 2013)

T=temperature, HMY=hydrogen molar yield, NI= no information

Compared to liquid-state fermentation and submerged fermentation technology which have been widely applied in the field of environmental engineering, SSF offers the advantages of lower energy requirements and less wastewater (Pandey, 2003). Even though it was once considered that SSF were only suitable for yeast and fungi due to their low water activity requirement, several bacterial cultures have been proven to be able to perform the SSF

processes (Pandey, Soccol and Mitchell, 2000; Sitthikitpanya, Reungsang and Prasertsan, 2018).



Pathways for the metabolism of pyruvate by *H. hydrogeniformans* based the genome annotation. (1) Lactate dehydrogenase (Halsa_1287), (2) Pyruvate formate lyase (Halsa_0723), (3) Pyruvate dehydrogenase (Halsa_0919, Halsa_2297, Halsa_0164), (4) Pyruvate ferrodoxin/flavodoxin oxidoreductase (Halsa_2334), (5) Phosphotransacetylase (Halsa_1556), (6) Acetate kinase (Halsa_1555).

A simplified metabolic pathway for glucose breakdown and the various metabolites produced using dark fermentation in *Clostridium*. Each arrow indicates one or several reactions in series along the pathways. In addition, the amount of hydrogen produced by the [Fe—Fe]-hydrogenase during the fermentation of each metabolite is also shown. Adapted from Chin et al. [29].

Figure 2.4 – A comparison of metabolism pathways of pyruvate by *H. hydrogeniformans* (left) and in *Clostridium* (right). Image reproduced from M. B. Begemann *et al.*, 2012 and Aly *et al.*, 2017.

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CHAPTER III

INVESTIGATION OF HYDROGEN-PRODUCING ABILITY OF EXTREMELY HALOTOLERANT BACTERIA FROM A SALT PAN AND SALT-DAMAGED SOIL IN THAILAND

3.1 Introduction

The dependence on fossil fuels for energy supply has had great impacts on global warming and climate change (Hansen and Sata, 2016). Therefore, the development of alternative renewable energy sources is being pursued globally (Show *et al.*, 2012). Biohydrogen is one of the promising candidates for future use because it is a CO_2-free, clean, and highly efficient energy carrier. Production of biohydrogen can be achieved through bio-photolysis, photo fermentation, and dark fermentation process (Nikolaidis and Poullikkas, 2017).

Dark fermentation process offers several advantages in industrial biohydrogen production. Among them are high production rates, high yields per mole of substrate, continuous production regardless of solar light condition (Dipasquale *et al.*, 2014), high variety of carbon sources as substrates, and has no oxygen limitation since the process is fully anaerobic (Hallenbeck, Abo-Hashesh and Ghosh, 2012; Boboescu *et al.*, 2016). On the other hand, dark fermentation also has several limitations, such as thermodynamically unfavorable condition as hydrogen yields increases and carbon dioxide's presence in the produced gas (Boboescu *et al.*, 2016).

Among carbon sources to supply fermentable sugars in biohydrogen production, lignocellulosic biomass is a highly considered option. It doesn't compete with food production, available abundantly in nature as grasses and woods, in forestry and agricultural residues, as well as in domestic and industrial wastes. It was estimated that lignocellulosic biomass residue is being produced more than 220 billion tons annually all over the world (Kumar *et al.*, 2015). However, lignocellulosic biomass requires pretreatment (Kumar *et al.*, 2015) such as alkaline and heat treatment followed by hydrolysis (with enzymes) prior to use as feedstock in fermentative hydrogen production (Phummala *et al.*, 2014). Most

bacteria do not retain their viability after alkaline and heat pre-treatment because of the high concentration of Na^+ ions, as the acidogenesis process in anaerobic digestion is severely inhibited by such conditions (Wang, Hou and Su, 2017). Therefore, an additional step to dilute or neutralize the alkaline conditions is required before proceeding to the next step of fermentative hydrogen production. However, this additional step makes the whole process less economical. One way to overcome this problem is by utilizing extremely halotolerant hydrogen-producing bacteria in dark fermentation process. These bacteria are advantageous for developing 'Next Generation Industrial Biotechnology' (NGIB) as they cut the costs of fresh water, oxygen, and sterilization (Chen and Jiang, 2018).

In the past few years, several studies have investigated hydrogen production using halotolerant and halophilic bacteria in dark fermentation process. Most of these studies focused mainly on pure cultures in a moderate halophilic environment with a salt concentration of 0.5 M (25 g/L) to 2.5 M (150 g/L), although one study also investigated mixed cultures. Liaw & Mah (Liaw and Mah, 1992) mentioned production of 144.5 µmol hydrogen from 5 mL medium with 12% NaCl and 0.5% glucose at 37°C by *Haloanaerobacter chitinovorans sp. nov.,* while Mouné et al. (Mouné *et al.*, 1999) found that *Halonanaerobacter salinarius sp. nov.* was able to produce 2mM hydrogen from 4.17 mM glucose substrate in 14-15% NaCl, 45°C, and pH 7.4-7.8. Matsumura et al. (Matsumura *et al.*, 2014) discussed the production of 1.7 mol H_2/mol mannitol by *Vibrio tritonius* strain AM2 at initial 2.25% (w/v) NaCl, pH 6 and 37°C.

Kivisto et al. (Kivistö, Santala and Karp, 2010) reported that *H. saccharolyticum* subspecies *saccharolyticum* produced 0.6 mol H_2/mol glycerol at a salt concentration of 150 g/L (2.6 M), pH of 7.4, temperature of 37 °C, and glycerol concentration of 2.5 g/L, while *H. saccharolyticum* subspecies *senegalensis* produced 1.6 mol H_2/mol glycerol at pH 7.0. Brown et al. (Brown *et al.*, 2011) found that *H. hydrogeniformans* was capable of producing hydrogen at a pH of 11, 7% (wt./vol.) NaCl, and 33°C. Pierra et al. (Pierra *et al.*, 2013) described the ability of a mixed culture affiliated to the family of *Vibrionaceae* to produce 0.9 ± 0.02 mol_{H2}/$mol_{glucose}$ at initial pH of 8 and temperature of 35°C

under a moderate halophilic environment (75 g/L NaCl). To date, no studies have investigated hydrogen production under conditions of a high salinity of 26% (6 M or 351.35 g/L NaCl).

This study aims to investigate hydrogen production by extremely halotolerant bacteria. The hypothesis is that extremely halotolerant hydrogen-producing bacteria can tolerate high concentrations of Na^+ ions. Based on this hypothesis, hydrogen production under different salinity concentrations before and after acclimatization was evaluated. The bacteria's requirement for chloride ions in high salinity conditions was also investigated.

3.2 Materials and methods

3.2.1 Seed microorganisms and medium

The soil samples were obtained from salt-damaged soil in Khon Kaen, Thailand and a commercial salt pan field near Bangkok (Samut Sakhon), Thailand. The soil samples were mixed with a substrate for cultivation in anaerobic conditions. The composition of the substrate used for biohydrogen production experiments at different salinity concentrations, i.e., between 3–10% and 15–26% NaCl, before the acclimatization experiments was as follows: 2 g/L $NaHCO_3$, 2 g/L K_2HPO_4, 1 g/L yeast extract, 0.7 g/L $(NH_4)_2HPO_4$, 0.75 g/L KCl, 0.85 g/L NH_4Cl, 0.42 g/L $FeCl_3 \cdot 6H_2O$, 0.82 g/L $MgCl_2 \cdot 6H_2O$, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.018 g/L $CoCl_2 \cdot 6H_2O$, 0.15 g/L $CaCl_2 \cdot 2H_2O$, and 0.018 g/L $NiCl_2 \cdot 6H_2O$. Glucose concentration were adjusted according to each experiment. All chemicals were purchased from Wako Pure Chemical Industries, Ltd., Japan. The composition of the substrate for the experiments on the bacteria's requirement for chloride ions in high salinity conditions, acclimatization period, and biohydrogen production at 26% NaCl after 2 years acclimatization was the same as that above, except that $NiCl_2 \cdot 6H_2O$ was omitted.

3.2.2 Culture conditions and experimental procedures

The first step of the experiment was to evaluate the hydrogen production in conditions of 3–10% salinity before acclimatization. The experiments were done

under six NaCl concentrations of 3%, 3.5%, 5%, 7%, 7.5%, and 10%. The second step of experiment was to evaluate the hydrogen production in conditions of 15–26% salinity before acclimatization and at 26% salinity after an acclimatization period of 2 years. The experiment was done at NaCl concentrations of 15%, 20%, and 26%.

The third step of the experiment was the evaluation of the bacteria's requirement for chloride ions in high salinity conditions. The experiment was done by comparing the culture under Na_2SO_4: NaCl ratios of 1:1 and 4:1. The ratio was prepared by weight to reach 26% (351.35 g/L) concentration of the mix. The third step was done after one year of acclimatization.

The experiments to determine biohydrogen production under salinity concentrations of 3–10% and 15–26% NaCl before acclimatization and various F/M ratio at 15% NaCl were done under the following conditions: 100 mL sealed serum bottles in a nitrogen atmosphere with an initial pH of 6.8 adjusted with 1 M HCl and 1 M NaOH and incubated in a shaking incubator (BT 100 & BT 300; Yamato Scientific Co., Ltd. Japan) at 35 °C and a 100-rpm shaking speed. Biogas samples were periodically collected, and the compositions were analyzed via gas chromatography.

The experiment to determine the bacteria's requirement for chloride ions in high salinity conditions followed the same conditions, except that 125-mL serum bottles were used. The main culture bottles with a volume of 500 mL each for the three different sources of soil were also maintained. These bottles were also used for acclimatization purposes. In acclimatization period of two years, the substrate's NaCl concentration was kept at 26%. Gas production was periodically measured, and substrate was changed after no gas production was detected. Anaerobic condition was maintained under nitrogen atmosphere.

Biohydrogen production at a NaCl concentration of 26% after 2-year period of acclimatization experiments was also studied under the same conditions, except that the initial pH was not adjusted, the serum bottles had a volume of 75 mL, and shaking incubator temperature was at 37°C.

3.2.3 Analytical method

The initial pH was adjusted by using a pH meter (D-13; Horiba Co. Ltd. Japan). The volume of the produced biogas was measured with a glass syringe. The composition of H_2, N_2, CH_4, and CO_2 was analyzed via gas chromatography (GC-8APT; Shimadzu Corp. Japan) with a 60/80 activated charcoal mesh column (1.5 m × 3.0 mm internal diameter) and Argon as a carrier gas, with operational temperatures of the injector, column, and detector of 50 °C, 60 °C, and 50 °C, respectively. The compositions of volatile fatty acids (VFAs) were determined by gas chromatography (GC-8APT; Shimadzu Corp. Japan) with a flame ion detector (FID) and a Unisole F-200 30/60 glass mesh column (3 m × 3.2 mm internal diameter). The operational temperatures for the injection port, FID detector, and oven were 250°C, 140°C, and 140°C, respectively.

The water content and total organic matter of the soil was determined via the JIS A 1203 test method for water content of soils and JIS A 1226-2000 test method for ignition loss of soils (Japanese Industrial Standards Association, 2000; Japanese Industrial Standard Association, 2009). Sample masses of 20.5506 to 44.1789 g were used to determine the soil's water content and ignition loss. Volatile Suspended Solid (VSS) was determined according to method 2540 E of *Standard Methods* (American Public Health Association, American Water Works Association and Water Environment Federation, 1999).

Soil salinity was determined by mixing soil and distilled water in 1:2.5 dry soil to water ratio, shaken for three hours at 180 rpm (Eyela Multishaker MMS; Tokyo Rikakikai Co, Ltd. Japan). After 25 minutes of settling time, the supernatant is measured by a thermo salinity meter (TS-391; As One Corp. Japan). The measurement results were then multiplied by the dilution factor of water content of soil.

3.2.4 Theoretical hydrogen production and yield

The theoretical hydrogen production was determined using equation (1) and (2) in Table 3.1. Based on equation (3.1), 1 mol of glucose will produce 4 mol of hydrogen. Thus, 1 g of glucose at standard temperature and pressure (STP)

conditions will produce 498 mL of H_2 via the acetic acid (HAc) pathway. Based on equation (3.2), 1 mol of glucose will produce 2 mol of hydrogen; hence, 1 g of glucose at STP conditions is required to produce 249 mL of H_2 via the butyric acid (HBu) pathway.

Theoretical maximum H_2 production reached is the comparison of observed cumulative H_2 in the experiments (mL) to the multiplication of glucose provided in the substrate (gr) with 498 mL of H_2 produced via the HAc pathway. Hydrogen molar yield (HMY) is the observed cumulative H_2 (mol) divided by glucose provided in substrate (mol).

Table 3.1 – Standard Gibbs energy of formation for glucose fermentation (obtained from (Thauer, Jungermann and Decker, 1977; Conrad, 1999))

Equations of fermentative reactions		ΔG^0
$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2$	(3.1)	-206 kJ
$C_6H_{12}O_6 + 2H_2 \rightarrow CH_3(CH_2)_2COOH + 2CH_3COO^- + H^+ + 2H_2$	(3.2)	-254 kJ
$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COO^- + 2H_2O + 2H^+$	(3.3)	-358 kJ
$C_6H_{12}O_6 \rightarrow 2CH_3CH(OH)COO^- + 2H^+$	(3.4)	-198 kJ
$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3CH_2OH + 2HCO_3^- + 2H^+$	(3.5)	-358 kJ

3.3 Results and discussion

3.3.1 Biohydrogen production at 3–10% salinity

Table 3.2 compares the biohydrogen production at salinities of 3% to 10% for a culture from salt-damaged soil from Khon Kaen, Thailand. Very low hydrogen production was observed at these salinity conditions. No methane was produced at salinities of 7.5% and higher. The experiments were conducted for 15 days with 0.15 g glucose for each 100-mL serum bottle. The maximum theoretical cumulative hydrogen yield was 74 mL H_2 for the acetic acid (HAc) pathway, and 37 mL H_2 for the butyric acid (HBu) pathway. The highest yield of 1.01 mol $H_2/mol_{glucose}$ was achieved at 10% salinity.

This is because the salt-damaged soil in its natural state is always exposed to a high salt concentration; thus, lower salt concentrations might not be suitable for extremely halotolerant anaerobic microorganisms to grow. Another reason for

this is that the food to microorganism (F/M) ratio at 1.5 mg/L volatile suspended solids (VSS)/mg/L of the substrate might not be ideal for production for 3-10% salinity conditions.

Table 3.2 – Biohydrogen production at 3–10% salinity of salt-damaged soil from Khon Kaen

Salt concentration (%)	Biohydrogen production (ml)	Theoretical maximum H_2 production reached (%)	HMY (mol H_2/ $mol_{glucose}$)
3	10.9	14.7	0.61
3.5	10.9	14.7	0.61
5	9.46	12.8	0.53
7	13.4	18.1	0.75
7.5	7.43	10	0.41
10	18.1	24.5	1.01
Glucose 0.15 g (5,000 mg/L), inoculum 3,000 mg/L VSS, F/M ratio 1.5			

Loss on ignition (LOI) is one of the most commonly used methods for quantifying soil organic matter (Hoogsteen *et al.*, 2015). The LOI results in Table 3.3 correspond to mixed sediment with low organic matter content obtained by Heiri et al. (Heiri, Lotter and Lemcke, 2001). Microbial biomass is usually low in salt-affected soils (Rath and Rousk, 2015). The Khon Kaen salt-damaged soil had a lower moisture content than the soil from the Samut Sakhon salt pan. Water content in soil is an important factor that influences the microbial activity of aerobic and anaerobic bacteria, and it also affects osmotic potentials of saline soils (Yan *et al.*, 2015).

Although LOI percentage and water content from Samut Sakhon salt pan showed a higher value, but the soil sample from the location gave lower biohydrogen yields, as shown in Table 3.5 and 3.7. One of the possible reason for this, is that high organic contamination doesn't occur in the salt pan since it was under a protected environment to maintain the purity of the salt, unlike the salt damaged soil of Khon Kaen. A major part of the organic matter that contributed to the LOI value in Samut Sakhon salt pan could be refractory organic. In Samut Sakhon salt pan's location, no plants and fish were observed, so not

many supply for organic matter were available.

Table 3.3 – Soil characteristics of Khon Kaen salt-damaged soil and soil from Samut Sakhon salt pan

	Water content (%)	**Ignition Loss after 600°C (g)**	**Loss on ignition (%)**	**Salinity (%)**
Khon Kaen salt damaged soil				
at the shore	32.94	0.42	2.37	7.74
close to the shore	21.33	0.25	1.57	20.16
farther from the shore	13.56	0.32	3.16	30.26
Samut Sakhon salt pan				
Salt pan surface	77.29	0.15	4.35	1.92
salt pan at 5 cm depth	60.4	0.95	5.41	3.45
Dry salt pan surface	43.26	0.04	0.43	7.80
Dry salt pan at 5 cm depth	40.15	0.29	1.51	6.15

3.3.2 Biohydrogen production at 15–26% salinity

The experiments on biohydrogen production at 15–26% salinity was performed twice. The first was done before acclimatization and the second was after two years of acclimatization. Table 4 compares biohydrogen production at the initial salinity of 15–26% before acclimatization. The highest production of 2.78 mol H_2/$mol_{glucose}$ occurred at 15% salinity. Theoretical hydrogen production value exceeding 100% of HBu pathway at 15% salinity suggested that the HAc pathway took place. Figure 3.1 shows that after a lag phase of 8 days, the cumulative hydrogen production at 15% salinity was significantly higher (49.8 mL (Table 3.2) than that at 10% salinity (18.1 mL (Table 3.2).

During the initial experiment, almost no hydrogen was produced under conditions of 20% salinity or more. After an acclimatization period of 2 years, confirmation experiments were conducted at 26% salinity (Table 3.5). The results showed that hydrogen production was possible at this concentration, with hydrogen yields of 0.66–1.15 mol H_2/$mol_{glucose}$.

Table 3.4 – Biohydrogen production at 15–26% salinity of salt-damaged soil from Khon Kaen

Salt Concentration (%)	Biohydrogen production (ml)	Theoretical maximum H_2 production reached (%)	HMY ($molH_2$/ $mol_{glucose}$)
15	49.8	67.3	2.78
20	0.02	0.03	0.00
26	0	0	0.00
Glucose 0.15 g (5,000 mg/L), inoculum 3,000 mg/L VSS, F/M ratio 1.5			

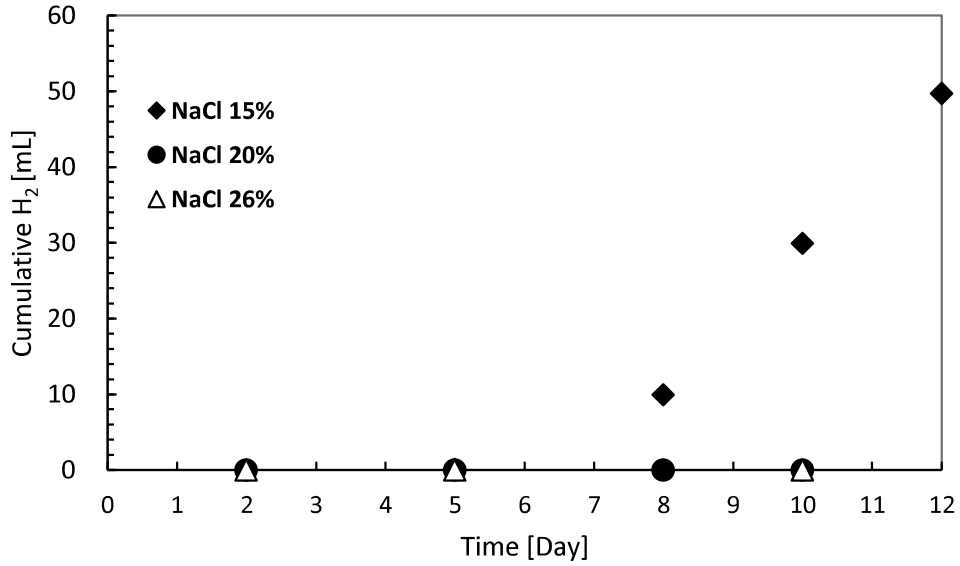

Figure 3.1 – Cumulative hydrogen production of salt-damaged soil in Khon Kaen

Khon Kaen salt damaged soil (1) was taken from the shore part of the pond, with finer soil and more water content. Khon Kaen salt damaged soil (2) was taken from the farther part of the pond, approximately 10 m apart toward drier land with coarser soil and less water content. Both samples were taken during the dry season. During the rainy season, the surfaces of both sampling locations are covered with water.

In Table 3.1, equations (3.1) and (3.2) show hydrogen production through the acetic acid and butyric acid pathways, respectively. Equations (3.3), (3.4), and

(3.5) show the pathways with no hydrogen production. The equations express the pathways of propionic acid fermentation, lactic acid, and alcohol fermentation, respectively.

Table 3.5 – Biohydrogen production at 26% salinity after two years of acclimatization

Soil Sample	Biohydrogen production (ml)	Theoretical maximum H_2 production reached (%)	HMY (mol H_2 /$mol_{glucose}$)
Samut Sakhon salt pan	13.44	27.02	1.08
Khon Kaen salt damaged soil (1)	14.31	28.76	1.15
Khon Kaen salt damaged soil (2)	8.22	16.53	0.66
Glucose 0.12 g (5,000 mg/L), inoculum 10 mL (3 mg/L VSS)			

The possible reason for the hydrogen yield being below the theoretical value is that the low F/M ratio produced conditions that were not optimum for the pathways expressed by equation (3.1) and (3.2). From the comparison of the standard Gibbs energy of the formation values, it can be assumed that the pathways for propionic acid fermentation (Eq. 3.3) and alcohol fermentation (Eq. 3.5) are more spontaneous than the rest. Thus, they are more likely to occur, as the substrate concentration was low, the reaction rate was high, and the hydrogen recovery rate was low. The production of propionate can decrease the production of hydrogen (Mosey, 1983; Shin, Youn and Kim, 2004; Li and Fang, 2007; Azwar, Hussain and Abdul-Wahab, 2014). Very low substrate concentrations can be unsuitable for hydrogen production as shown in Table 3.6. Figure 3.2 showed the composition of VFAs produced for each F/M condition in Table 3.6. For F/M ratio of 1.5 and 2, almost all propionic acid and butyric acid were transformed to acetic acid. Optimization of the substrate's composition should be considered in future work to increase the hydrogen molar yield.

Table 3.6 – Biohydrogen production at 15% salinity experiments for 0.5-2.0 F/M ratio of salt-damaged soil from Khon Kaen before acclimatization

F/M ratio	Glucose (g)	Glucose (mg/L)	Biohydrogen Production (ml)	Theoretical maximum H_2 production reached (%)	HMY (mol H_2/ $mol_{glucose}$)
0.5	0.045	1,500	8.26	36.9	1.48
1	0.09	3,000	24.49	54.7	2.19
1.5	0.15	5,000	49.75	67	2.67
2	0.18	6,000	5.31	1.34	0.24
Inoculum 3,000 mg/L VSS					

3.3.3 Evaluation of the bacteria's requirement for chloride ions in high salinity conditions

In this experiment, Na_2SO_4 was used to partially replace NaCl in two different ratios (1:1 and 4:1) to confirm the extremely halotolerant hydrogen-producing bacteria's requirement for Cl^- ions. The substrate was adjusted such that the overall salinity of the mixture of Na_2SO_4 and NaCl was 26%. The bacteria's requirement for Cl^- ions was confirmed by a clear difference in the amount and yield of hydrogen production at different ratios of Na_2SO_4 to NaCl (Figure 3.3 and Table 3.7).

Halophilic archaea and halophilic fermentative bacteria use the 'salt-in' strategy in their survival mechanism in extremely hypersaline conditions (Roberts *et al.*, 2005; Kivistö and Karp, 2011). To adapt to this condition, cells maintain high salt concentrations at the intracellular level to sustain isosmotic conditions within the cell. Usually, K^+ and Cl^- ion salts are accumulated in molar concentrations at the intracellular level (Kivistö and Karp, 2011). Cl^- is the preferred anion accumulated in the 'salt-in' strategy and it is possible that it plays critical roles in haloadaptation (Roberts *et al.*, 2005); however, some halophilic microorganisms also utilize sulfate in high concentrations (Ede, Hafner and Fredericks, 2004; Kivistö and Karp, 2011). The energy for outward transport of Na^+ ions is provided through the H^+ gradient in the electrogenic Na^+/H^+ antiporters, while K^+ and Cl^- enters through a symporter system in response to the cell's membrane potential (Lanyi, 1979; Oren, 2001).

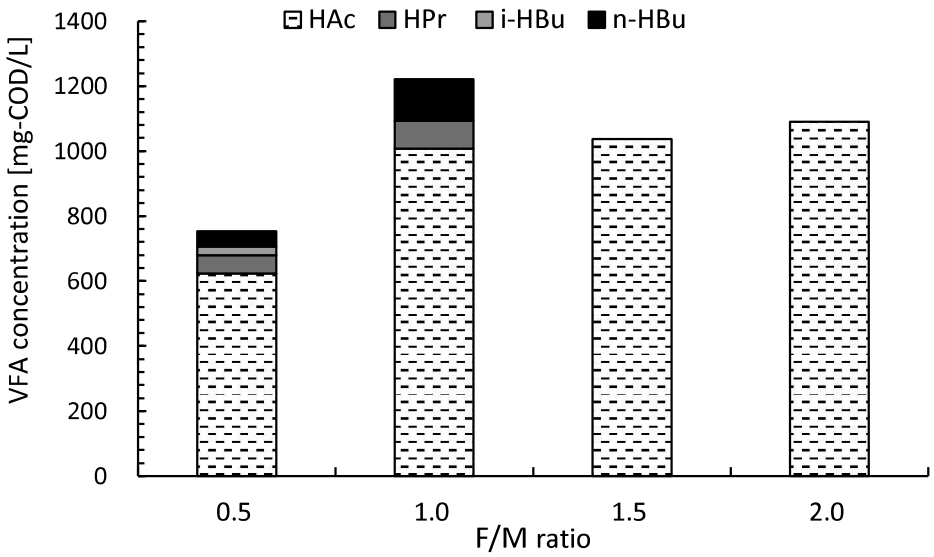

Figure 3.2 – VFA composition for 0.5-2 F/M ratio at 15% salinity experiments of salt-damaged soil from Khon Kaen before acclimatization (HAc= acetic acid, HPr = propionic acid, i-HBu = isobutyric acid, n-HBu = n-butyric acid)

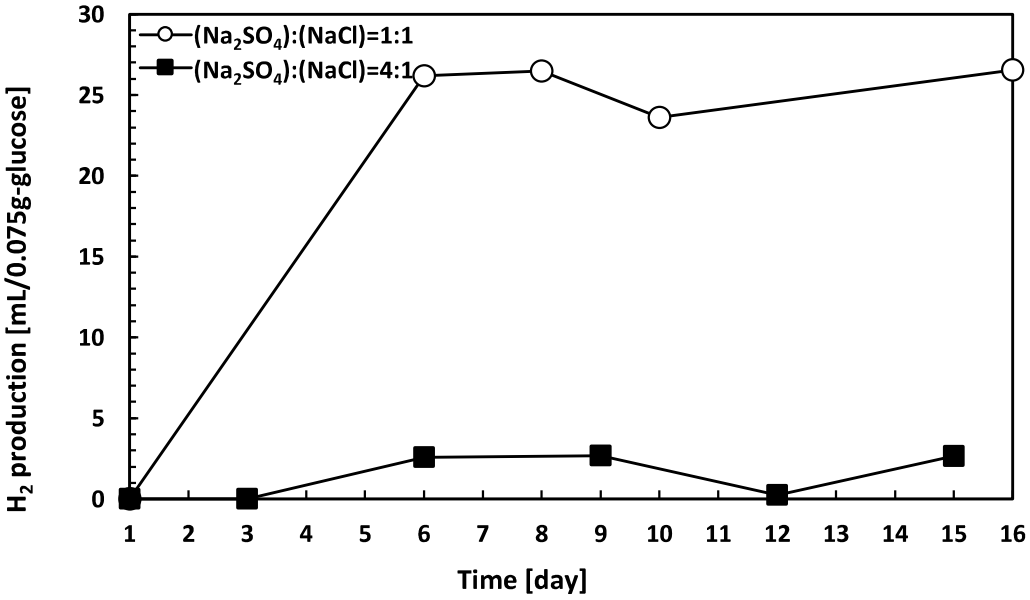

Figure 3.3 – Cl^- ion requirement for H_2 production in salt-damaged soil of Khon Kaen

Approximately one equivalent unit of adenosine triphosphate (ATP) will be needed to accumulate 1.5 to 2 molecules of KCl (Oren, 2001). This mechanism

clearly shows a high requirement for K^+ ion, which the substrate might have not supplied in sufficient quantity compared to Na^+ and Cl^- ions (Figure 3.4). Only 1.3 g/L of K^+ was available in the substrate, compared to 351.35 g/L of NaCl.

In non-halophilic bacteria, high sulfate concentration suppressed hydrogen production by shifting the metabolic pathway from butyrate fermentation to ethanol. The decrease may also be caused by the toxicity of hydrogen sulfide (Lin and Chen, 2006). To further confirm this result, another experiment with only Na_2SO_4 salt is suggested for the future work. Hydrogen consumption by sulfate reducing bacteria might be negligible for this experiment due to the near saturation NaCl concentration that limited the growth of such bacteria.

Table 3.7 – Biohydrogen production at different ratios of Na_2SO_4 to NaCl

Soil Sample	(Na_2SO_4): (NaCl)	Glu-cose (g)	Glu-cose (mg/L)	Inocu-lum (mg/L VSS)	Bio-hydrogen production (ml)	Theoretical maximum H_2 production reached (%)	HMY (mol H_2/ $mol_{glucose}$)
Samut Sakhon salt pan	1:1	0.022	7,500	5,000	1.77	16.0	0.64
Khon Kaen salt damaged soil (1)		0.0044	2,250	1,500	1.57	71.2	2.85
Khon Kaen salt damaged soil (2)		0.0044	2,250	1,500	0.26	23.3	0.47
Samut Sakhon salt pan	4:1	0.075	1,500	1,000	0	0	0.00
Khon Kaen salt damaged soil (1)					2.66	7.12	0.29
Khon Kaen salt damaged soil (2)					0	0	0.00

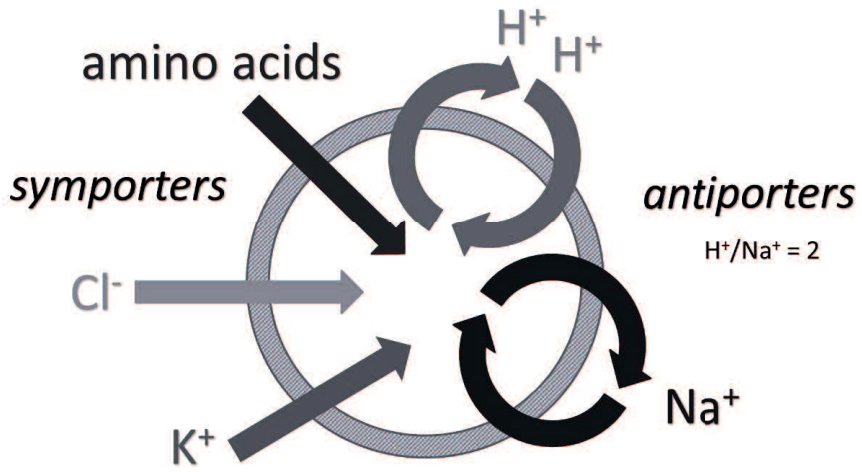

Figure 3.4 – Na^+ and Cl^- roles in transport process of halophilic bacterial membrane

3.4 Summary

The experimental results showed that it is possible to produce biohydrogen under high salt concentrations (26% NaCl) after at least one year of acclimatization. This indicates that extremely halotolerant hydrogen-producing bacteria can exist under such concentrations. The best hydrogen molar yield in this study was 2.85 mol H_2/mol glucose which was produced at 13% NaCl, initial pH 6.8, 35°C, and food to microorganism ratio of 1.5 after 1 year of acclimatization.

Acknowledgments

The authors would like to thank Kyoko Ikeda and Hana Watari for their valuable help. The first author would like to gratefully acknowledge the Indonesian Endowment Fund for Education (LPDP) and The Directorate General of Higher Education (DIKTI) for providing BUDI-LN scholarship. This work was supported by JSPS Core-to-Core Program, A. Advanced Research Networks which was financially supported by Japan Society for the Promotion of Science (JSPS).

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CHAPTER IV

OPTIMUM CONDITIONS FOR BIOHYDROGEN PRODUCTION BY EXTREMELY HALOPHILIC BACTERIA FROM THE SALT PAN OF SAMUT SAKHON, THAILAND

4.1 Introduction

As the demand for energy continues to grow, several countries worldwide have taken the measures to shift to a hydrogen economy and society, and others have started taking action according to the hydrogen fuel initiative. Currently, renewable sources only contribute to 4% of the world's total hydrogen production (Parthasarathy and Narayanan, 2014), the other 96% of hydrogen production comes primarily from fossil fuel sources (Alhamdani *et al.*, 2017), such as natural gas reforming and coal gasification. As the global trend moves towards renewable energy sources, hydrogen production should follow the lead. Among several methods to produce hydrogen from renewable sources by biological pathways, biohydrogen production by dark fermentation has been considered as a practically feasible option (Łukajtis *et al.*, 2018).

Lignocellulosic biomass, e.g., woods and grasses found in nature, is available in great quantities as biomass resource for dark fermentation, as residues from forestry, food and agricultural industries and in municipal solid wastes (Kumar *et al.*, 2015; Bundhoo, 2019). However, the structure of lignocellulosic biomass, which consists of cellulose, hemicellulose, and lignin, is naturally recalcitrant. Hemicellulose and cellulose can be biologically utilized after the application of several stages of unit processes, as follows (Kim, Lee and Kim, 2016): size reduction through mechanical means, pretreatment to make cellulose more readily available for enzyme conversion, enzymatic saccharification of cellulose and hemicellulose to hydrolyze into fermentable sugars, microbial fermentation involving conversion of fermentable sugars into fuels by bacteria, and product recovery and purification. Among those processes, pretreatment has been considered as one of the most costly (Den *et al.*, 2018), although alkaline

pretreatment with NaOH is considered as the most widely used and cost-effective for lignocellulosic biomass (Kim, Lee and Kim, 2016).

Extremely halophilic bacteria can reduce water use in the dark fermentation process of pretreated lignocellulosic biomass since they're capable of producing hydrogen under high conditions of NaCl. After alkaline pretreatment with NaOH, followed by neutralization with HCl to a suitable pH condition, fermentation processes to produce hydrogen can continue readily without the need for washing the pretreated lignocellulosic material with water. The anaerobic process can also help to cut the cost of electricity and devices needed for aeration. The halophilic environment is beneficial for preventing the growth of hydrogenotrophic methanogens, which have never been reported to occur at the salt concentration of 120 g/L or more (Oren, 2011).

Biohydrogen production by halophilic bacteria has been reported in several previous studies. Most halophiles capable of producing biohydrogen have been reported to be from the *Halanaerobium* genus. For example, *Halanaerobium hydrogeniforms* has been described to produce hydrogen up to 2.42 mmol H_2/mmol glucose (Begemann *et al.*, 2012). Meanwhile, *H. salinarius* could produce 2 mM hydrogen from 4.17 mM glucose substrate in 14-15% NaCl at pH 7.4-7.8 (Mouné *et al.*, 1999). Additionally, *H. chitinovorans* was reported to have the ability to produce 144. 5 µmol H_2 from 5 mL 0.5% glucose medium at 37°C and 12% NaCl (Liaw and Mah, 1992). Hydrogen production from glycerol by *H. saccharolyticum* subspecies *senegalensis* and *saccharolyticum* were reported to give a yield of 1.6 and 0.6 mol H_2/mol glycerol, respectively (Kivistö, Santala and Karp, 2010). Very few studies have reported biohydrogen production in extremely halophilic condition (200-300 g/L salinity) with mixed culture.

This study aimed to determine the optimum pH, temperature, and salinity for biohydrogen production from glucose by extremely halophilic bacteria isolated from the Samut Sakhon salt pan, in preparation for biohydrogen production from lignocellulosic biomass. Additionally, the ability of the bacteria to ferment various types of carbohydrates was examined, and identification of the predominant hydrogen producer(s) among the acclimatized mixed culture of the Samut Sakhon salt pan was attempted.

4.2 Materials and methods

4.2.1. Inoculum and substrate

Microorganisms collected from the soil of a commercial salt pan field in Samut Sakhon, Thailand and cultivated in a 500 mL serum bottle under 26 wt% NaCl (6 M or 351.35 g/L) were used as a seed inoculum. The substrate for biohydrogen production experiments was prepared as described by Taroepratjeka et al. (Taroepratjeka *et al.*, 2019) with the exclusion of $NiCl_2 \cdot 6H_2O$. The glucose concentration was adjusted at 10 g/L. All chemicals used in this study were acquired from Wako Pure Chemical Industries, Ltd. (Japan), except where otherwise stated.

Due to the opacity of the mixed culture, it was not possible to observe the bacteria's concentration through optical density method. Therefore, a different method to approximate the seed inoculum's growth phase was employed. The substrate of the cultivation bottle was changed periodically by removing the suspension with a glass syringe and separate the biomass and the liquid through centrifugation at 12000 rpm, 25°C, for 10 minutes (Himac CF15RN; Hitachi Koki Co. Ltd., Japan). The supernatant was discarded, while the biomass pellets were returned to the cultivation bottle along with the fresh substrate. The cultivation bottle was kept at 37° C in an incubator (MIR-262; Sanyo Electric Co. Ltd; Japan).

After the substrate change, biogas production in the cultivation bottle was measured and removed daily with a glass syringe. A biogas production pattern was established over time. The peak biogas production was reached after 12 days; therefore, the microbial suspension was used as an inoculum for hydrogen production experiment at day 13.

4.2.2. Batch experiment for hydrogen production

Batch experiments were carried out in 70 and 75 mL serum bottles in duplicates for each condition. Each serum bottles were filled with 15 mL inoculum from the cultivation bottles plus 15 mL of the substrate. The bottles were capped with butyl rubber stoppers and aluminum caps, and the headspace was replaced

with nitrogen gas. The experiments to determine optimum pH and temperature were performed in the presence of 26% NaCl. The initial pH was measured with a pH meter (D-51; Horiba Co. Ltd. Japan) and adjusted with NaOH (0.1, 1, or 3 M) and HCl (0.2 M).

To determine the optimum pH condition, the pH of the substrate was adjusted to 7.5, 8, 9.0, 9.5, or 10. The shaking incubator (BT 300; Yamato Scientific Co. Ltd., Japan) was set at 37°C with a shaking speed of 100 rpm. For analysis of the optimum temperature, the pH was adjusted to 9.0. The temperatures of the shaking bath incubators (BT 100 and BT 300; Yamato Scientific Co. Ltd., Japan) were set at 35°C, 37°C, 40°C, 42°C, or 45°C. For analysis of the optimum salt concentration, the substrates were prepared with 15%, 20%, or 26% NaCl. The pH was adjusted to 9.0, and the temperature was set to 35°C.

The volume of the produced biogas from each vial was measured and removed periodically with 5-to 50-mL glass syringes. H_2, N_2, CH_4, and CO_2 gas compositions in the vials were measured following volume measurement with gas chromatography (GC-8APT/TCD; Shimadzu Corp., Japan), using a 60/80 activated charcoal mesh (Shimadzu Corp., Japan). Argon was used as a carrier gas at the pressure of 600 kPa. The operational temperatures were 50°C for injector, 60°C for column, and 50°C for the detector, with the current set at 80 mA.

The hydrogen gas volume was calculated to STP volume by multiplying the measured gas volume by the dry biogas factor for the corresponding temperature (Equation 4.1) according to the method proposed by Richards (Richards *et al.*, 1991).

$$V_0 = DBF_t \times V_t \qquad (4.1)$$

where

V_0 = dry (non-water) biogas volume at 0°C (L)

DBF_t = dry biogas factor (L dry at 0°C (L measured at C)$^{-1}$)

V_t = biogas volume measured at temperature T (L)

The dry biogas factor for this work are provided in Table 4.1. To simplify the calculation, a regression for temperature versus dry biogas factor was performed:

$$DBF_t = 1.0513 - 0.00061 \times T \quad (4.2)$$

where

T = biogas temperature (0°C) within the range of 35 to 46°C.

Table 4.1– Dry biogas factor for gas volume calculation to STP (from Richards *et al.*, 1991)

Biogas temperature		Saturated steam volume (ft^3 lb^{-1})	Water vapor density (gL^{-1})	Water vapor volume ($1L^{-1}$)	Dry biogas volume ($1L^{-1}$)	Dry biogas factor (1@0°C L^{-1})
°F	°C					
95	35.0	404.4	0.0396	0.0556	0.9444	0.8371
100	37.8	350.4	0.0457	0.0648	0.9352	0.8216
105	40.6	304.5	0.0526	0.0752	0.9248	0.5052
110	43.3	265.4	0.0604	0.0870	0.9130	0.7879
115	46.1	231.9	0.0691	0.1005	0.8995	0.7696

Volatile fatty acids (VFAs) were measured with gas chromatography (GC-8APF/FID; Shimadzu Corp. Japan) and a Unisole F-200 30/60 glass mesh column (Shimadzu Corp., Japan). The VFAs were measured against a standard consisted of 1000 mg/L COD each of acetic acid, propionic acid, isobutyric acid, n-butyric acid, isovaleric acid, and n-valeric acid. Nitrogen was used as a carrier gas at the pressure of 600 kPa. The operational temperatures were 250°C for injector, 140°C for column, and 140°C for detector, with the current set at 80 mA. Volatile Suspended Solids (VSS), which represented the microorganism concentration in the sample were determined according to *Standard Methods* (American Public Health Association, American Water Works Association and Water Environment Federation, 1999).

4.2.3. Carbohydrate fermentation test

Phenol red fermentation broth was used to determine the microorganism's ability to ferment carbohydrates and consisted of 10 g/L trypticase peptone (BBL,

Becton Dickinson, USA), 1 g/L beef extract (MP Biomedicals, Inc., USA), 0.018 g/L phenol red (7.2 ml of 0.25% phenol red solution), 10 g/L carbohydrate, and 351.35 g/L NaCl, made up in distilled water. The salinity of the broth was adjusted to 26% NaCl to support the acclimatized seed microorganisms. The broth without carbohydrates was prepared as a 1.25-fold solution and autoclaved (HA-300MIV, Hirayama Manufacturing Corp., Japan). Carbohydrates were prepared separately and added to the autoclaved broth using 0.2-µm syringe filters (Minisart RC, Sartorius AG, Germany), except for starch and α-cellulose.

Each carbohydrate was tested in triplicate in 10-mL serum bottles under a nitrogen headspace. For each set of carbohydrates, one negative control with no addition of seed microorganisms was also prepared. The fermentation was observed up until three weeks of incubation, owing to the slow growth of the extremely halophilic anaerobic fermenter bacteria. Phenol red color change from red to yellow indicated positive carbohydrate fermentation. Biogas production was measured with a glass syringe from each bottle after the color change.

4.2.4. Theoretical hydrogen production and hydrogen molar yield

Theoretical hydrogen production was based on these following equations for acetic acid (HAc) and butyric acid (HBu) pathways:

$$\mathbf{C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2} \quad (4.3)$$

$$\mathbf{C_6H_{12}O_6 + 2H_2O \rightarrow CH_3(CH_2)_2COO^- + 2HCO_3^- + 3H^+ + 2H_2} \quad (4.4)$$

Following equation (4.3), 4 mol hydrogen will be produced from 1 mol glucose. Hence, 498 mL of hydrogen will be produced from 1 g glucose at standard temperature and pressure (STP) conditions through the HAc pathway. For the HBu pathway in equation (4.4), 2 mol hydrogen will be produced from 1 mol glucose. Therefore, 249 mL H_2 will be produced from 1 g glucose through the HBu pathway at STP conditions.

The efficiency of the fermentative process is compared with the hydrogen molar yield (HMY), which is the value of cumulative hydrogen produced (mol H_2) divided by glucose provided in the substrate (mol glucose). Theoretically, the

maximum HMY for glucose in anaerobic fermentation is 4, which can be reached through the HAc pathway (Equation 1).

4.2.5. PCR-DGGE

Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) was performed according to the methods of Kongjan *et al.* (Kongjan *et al.*, 2010) and Schäfer and Muyzer (Schäfer and Muyzer, 2001). Genomic DNA was extracted and purified with an ISOIL for Beads Beating kit (Nippon Gene Co., Ltd., Japan). Two-step nested PCR was used to ensure species contributing less than 1% to the total population could be detected with DGGE.

The first step of the PCR to amplify the 16s rRNA was carried out using the universal bacteria primers of 27F and 1492R (Eurofins Genomics Inc., Japan). EmeraldAmp PCR Master Mix (Takara Bio Inc., Japan) was used to prepare the PCR mixture. Following PCR, the products were checked with electrophoresis (Mupid-2plus system, Mupid Co., Ltd., Japan) with 1% Agarose (L03, Takara Bio Inc., Japan), and analyzed using a ChemiDoc XRS system (Bio-Rad Laboratories, USA).

The amplicon from the first step of PCR was used as a DNA template in the second step of PCR to amplify the V3 region of the 16s rRNA with primer pairs 341F-GC and 518R. The PCR product of the second step of PCR was electrophoresed on 2% Agarose S (Nippon Gene Co., Ltd., Japan.), and analyzed.

DGGE analysis using the PCR amplicon obtained from the second step was performed using a DCode Universal Mutation Detection system (Bio-Rad Laboratories, USA) with 8% (v/v) polyacrylamide gels and a denaturant gradient of 30-70% (100% denaturing solution contained 7 M urea and 40% [v/v] formamide). Electrophoresis was performed for 16 h at 70 V and 60°C in 1 × TAE buffer. SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Thermo Fisher Scientific, USA) was used to stain the polyacrylamide gel, which was then analyzed on a ChemiDoc XRS system (Bio-Rad Laboratories, USA) and documented with Quantity One software (version 4.6.5; Bio-Rad Laboratories, USA).

Several bands that were considered important were excised from the gel and incubated with 50 µL nucleic acid-free water at 4°C overnight. The supernatants were used as a DNA template and re-amplified with 341F and 518R primer pairs. Following PCR, the amplicons were cleaned using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co, Germany) and sent to Yamaguchi University Center for Gene Research for Sanger sequencing. Alignments for phylogenetic analysis were made using MUSCLE. NCBI's MEGABLAST was used to identify the closest match for the V3 region of the 16s rRNA's gene sequence (194 bp) in the GenBank data base. A phylogenetic tree was constructed using the neighbor-joining method using the MEGAX software. The bootstrapping analysis was carried out with 1000 replicates to estimate the confidence of tree topologies.

Later on, the inoculum was grown on a GYP agar medium specified by Kobayashi *et al.* (Kobayashi, Kimura and Fujii, 2000) to isolate and purify the species. This measure was taken to confirm the MEGABLAST result of the PCR-DGGE. PCR of 16S RNA with 27F and 1492R primers and Sanger sequencing was then performed to colonies growing on the GYP agar. The EZBioCloud database for 16S RNA was used as a comparison for the BLAST result.

4.3 Results and discussion

4.3.1. Optimum conditions for biohydrogen production

Biohydrogen production and HMY for different temperatures and pH conditions in the presence of 26% NaCl are shown in Table 4.2. Only very little biohydrogen was produced at 37°C and pH 9.5 or 10. However, at pH 7.5, 8, and 9, the HMY and cumulative biohydrogen production did not differ much for conditions with the same salinity and temperature (Figure 4.1). In a separate experiment, HMY at pH 8.5 was also confirmed to be similar to that at pH 9.0. The highest HMY at 37°C and 26% NaCl was observed at pH 9 (1.20 mol H_2/mol glucose). Therefore, subsequent experiments for identification of the optimum temperature and salinity were performed at pH 9.0. The VFAs measurements

also showed the highest acetic acid and butyric acid production at pH 9.0 (Figure 4.3), which corresponded with the HMY values. As a comparison, extreme halophiles such as *H. lacusrosei*, which can live in NaCl concentrations of 7.5–34%, show optimum growth at pH 7 (Cayol *et al.*, 1995). On the other hand, *H. alcaliphilum* can live under conditions of pH values up to 10; however, the range for NaCl concentrations is only 2.5–25% (Tsai *et al.*, 1995).

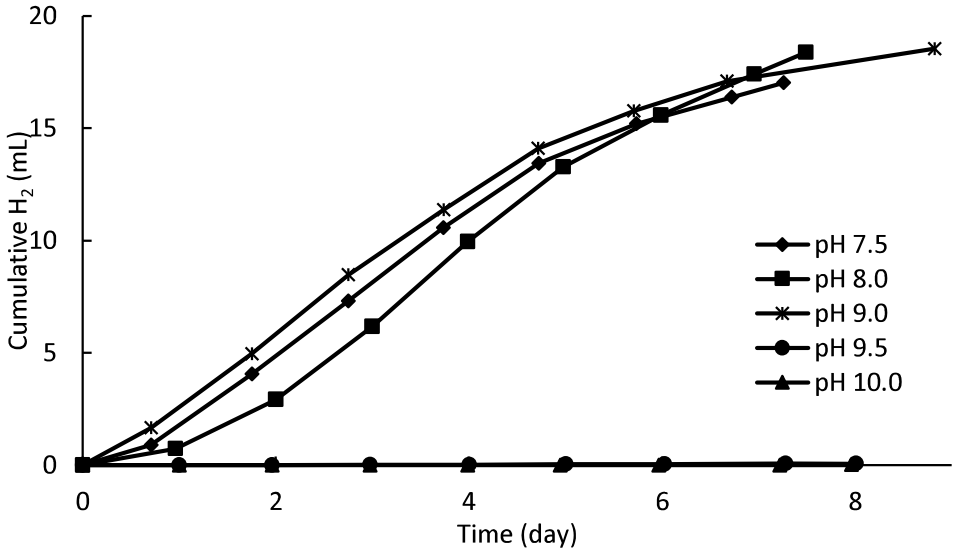

Figure 4.1 – Cumulative biohydrogen production at different pH conditions (Temperature 37°C, 26% NaCl. Volume was measured as STP)

The cumulative hydrogen results' difference between pH 9.0 and pH 9.5 can be explained in Figure 4.2, where NaOH must be added in considerably high amount (51.6 mg) for 100 mL of substrate, compared to the adjustment between other pH values to raise the pH between 9.0 to 9.5. A study showed that table olives fermentation process that included 1% (w/v) NaOH treatment for debittering has a negative effect on the halophilic population of the ecological community compared to the untreated olives (Cocolin *et al.*, 2013). Figure 4.3 also showed a drop of volatile fatty acids production between pH 9.0 to pH 9.5.

In this experiment, the highest HMY of 1.45 mol H_2/mol glucose was produced under the conditions of 26% NaCl (351 g/L), 35°C, and pH 9 (Table 4.2). Compared to previous studies (Table 4.3), this study demonstrated the result

which achievable with the highest concentration of NaCl which to date has never been reported. While the result was below the highest published HMY for halophiles was 2.42 mol H_2/mol glucose produced by *H. hydrogeniformans* in the presence of 70 g/L NaCl (Begemann *et al.*, 2012), this value was considerably higher than the amount of biohydrogen produced by mixed culture reported by Pierra *et al.* (Pierra *et al.*, 2013) at 0.9 ± 0.02 mol H_2/mol glucose (Table 4.3).

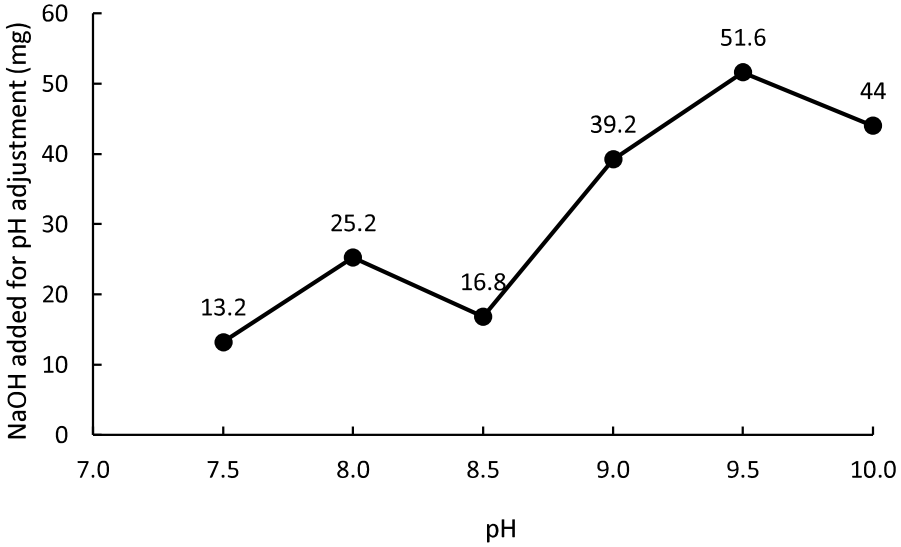

Figure 4.2 – NaOH mass (in milligram) added for pH value adjustment in 100 mL of substrate

Overall, the HMY values produced by halophilic bacteria values were considerably lower than the highest value reported through dark fermentation (3.9 mol H_2/mol glucose) by *Enterobacter cloacae* (Mandal, Nath and Das, 2006). The high HMY produced by *Enterobacter cloacae* was reached using a strategy in which the H_2 partial pressure was decreased from 760 mm Hg to 380 mm Hg in the head space of a batch fermentation reactor (Mandal, Nath and Das, 2006). Biohydrogen synthesis pathways are prone to high partial pressures of H_2, and when end-product inhibition occurs, the pathway will shift away from biohydrogen production to lactate, ethanol, or butanol production (Levin, Pitt and Love, 2004).

Table 4.2 – Biohydrogen production at various pH, temperature and NaCl concentration

NaCl	T (°C)	pH	Inoculum concentration (mg-VSS/L)	Biohydrogen production (mL)	Theoretical maximum H_2 production reached (%)	HMY (mol H_2/ mol glucose)
26%	37	7.5	334	17.02	23%	0.91
26%	37	8.0	334	18.37	25%	0.98
26%	37	9.0	334	18.55	25%	0.99
26%	37	9.5	334	0.07	0%	0.00
26%	37	10	334	0.01	0%	0.00
26%	35	9.0	452	24.59	33%	1.32
26%	40	9.0	452	21.55	29%	1.16
26%	42	9.0	452	19.79	26%	1.06
26%	45	9.0	452	20.60	28%	1.10
26%	35	9.0	432	26.97	36%	1.45
20%	35	9.0	432	23.57	32%	1.26
15%	35	9.0	432	21.88	29%	1.17

Fermentation period: 5-9 days. Volume was measured as STP

Figure 4.4 shows the cumulative biohydrogen values at different temperatures. The highest cumulative hydrogen production was produced at 35°C. While hydrogen production for halophiles were mostly reported between the range of 33 to 37°C (Table 4.3), this experiment showed that hydrogen production was possible at higher temperature of 40 to 45°C, with the steepest curve at 42°C.

Experiments with lower NaCl concentrations (15% and 20%) yielded steeper curves at the beginning but eventually resulted in lower cumulative hydrogen and HMY than that in the presence of 26% NaCl, (Figure 4.5 and Table 4.2). These results could be related to the pre-adaptation of the bacteria to high NaCl concentrations. Extremely halophilic bacteria manage to live in the presence of high salt concentrations by maintaining a high intracellular KCl concentration (Oren, 2013).

Optimization of salinity and temperature for shorter hydraulic retention time to cut operating cost in reactors is suggested for the future work.

4.3.2. Carbohydrate fermentation test

The acclimatized seed microorganisms from Samut Sakhon salt pan were capable of fermenting various types of hexoses, pentoses, dissacharides, and starch, as well as glycerol (Table 4.4).

Lignocellulosic-derived sugars include glucose, galactose, mannose, xylose, arabinose, fucose, and rhamnose (Mosier *et al.*, 2005; Jönsson and Martín, 2016). The fermentation test results indicated that the seed microorganisms were able to ferment these types of sugars. The microorganisms' ability to produce a copious amount of biogas from d-fructose suggested a potential ability to produce biohydrogen from food waste. A study of biohydrogen production form lignocellulosic and food waste biomasses by the acclimatized bacteria through anaerobic solid-state fermentation is suggested for future work.

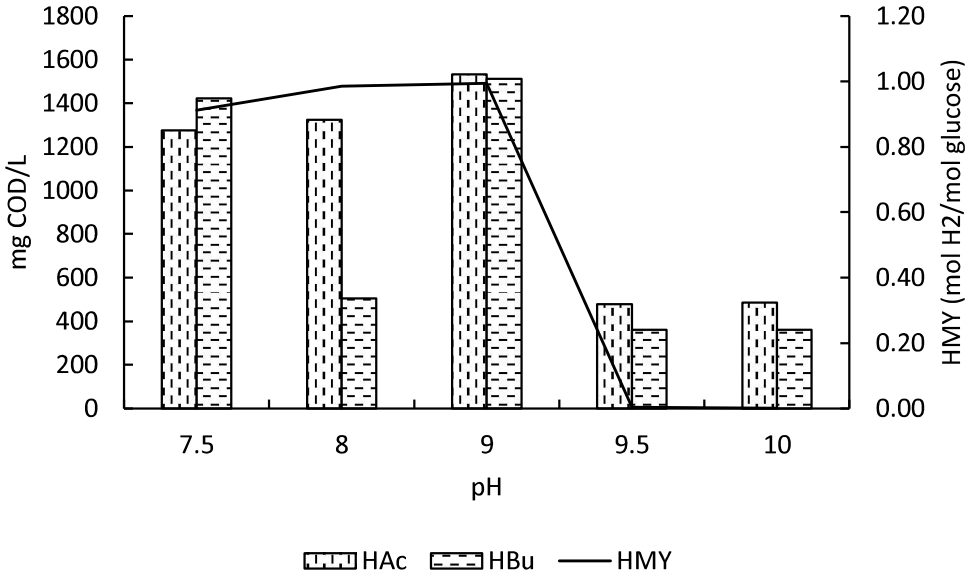

Figure 4.3 – Volatile Fatty Acid composition at different pH conditions (HAc = acetic acid, HBu = butyric acid, HMY = hydrogen molar yield). Temperature 37°C, 26% NaCl. Volume was measured as STP.

4.3.3. Identification of hydrogen producer of the acclimatized mixed culture from Samut Sakhon, Thailand

The strongest band in DGGE analysis were band 1 and 2 (Figure 4.6). Excised bands 1 and 2 showed the same sequencing results. For the V3 region of 16s rRNA sequences, the NCBI's MegaBLAST confirmed 100% similarity of these bands with *H. fermentans* strain R-9. The phylogenetic tree showing the relationship between the sequence of the excised bands and other *Halanaerobium* species is shown in Figure 4.7. Colony PCR of 16s rRNA, which was later performed to confirm these results, showed 99.3% similarity with *H. fermentans* strain R-9 using NCBI's MegaBLAST and 99.36% similarity using EzBioCloud. The resulting sequence of colony *H. fermentans* strain B4 (1424 bp) has been submitted to the GenBank data base (Accession number MN133965).

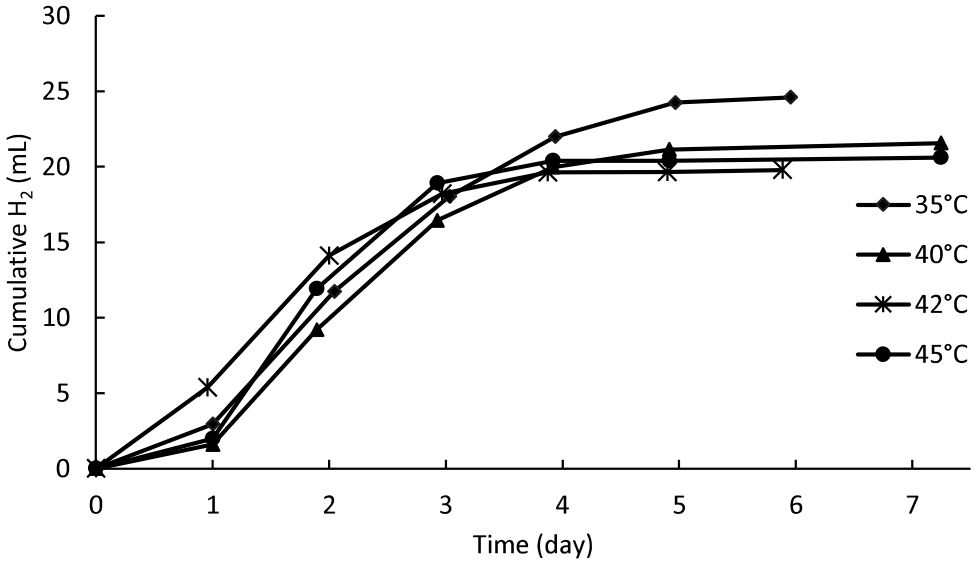

Figure 4.4 – Cumulative biohydrogen production at different temperature conditions (pH 9, NaCl 26%. Volume was measured as STP)

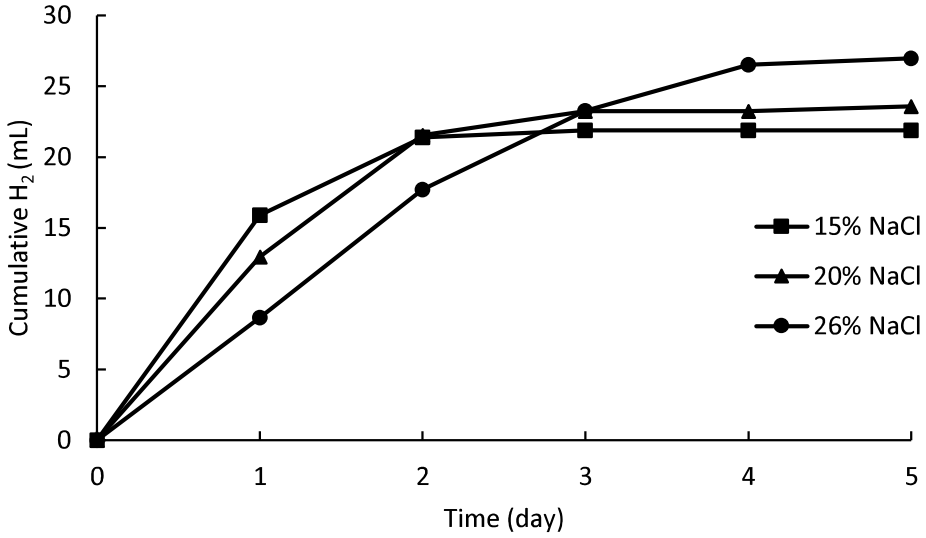

Figure 4.5 – Cumulative biohydrogen production at different NaCl concentrations (Temperature 35°C, pH 9. Volume was measured as STP)

H. fermentans was first isolated from salted puffer fish ovaries in Japan (Kobayashi, Okuzumi and Fujii, 1995; Kobayashi, Kimura and Fujii, 2000). This gram-negative, motile, strictly anaerobic bacteria species was also reported to be present in other salted food products from other countries including *yegyo ngapi* from Myanmar (Kobayashi *et al.*, 2016), *pla-ra* from Thailand, *pa-daek* from Laos (Marui *et al.*, 2015), salted and fermented seafood from Korea (Kim and Park, 2014). Fermentation products of *H. fermentans* include acetate, formate, lactate, ethanol, H_2, and CO_2 (Kobayashi, Kimura and Fujii, 2000).

Although other bacteria in the mixed culture could not be identified yet in this study, these bacteria played a substantial role in fermentation. The mixed culture was capable of fermenting L-arabinose in repeated tests; however, pure cultures of H. fermentans did not have this ability in our study. This difference in carbohydrate utilization ability was also observed for L-rhamnose, starch, and glycerol, which were fermentable by the mixed culture, but showed negative results in the report by Kobayashi et al. (Kobayashi, Kimura and Fujii, 2000). Table 4.4 shows a comparison of carbohydrate fermentation ability between mixed culture from this study and pure H. fermentans strain R-9 culture. The VFA measurements also showed that butyric

acid, a metabolite which is reported not to be produced by H. fermentans (Kobayashi, Kimura and Fujii, 2000), was produced by the mixed culture (Figure 2). This phenomenon demonstrated the benefits of mixed culture utilization in the fermentation of waste-derived carbohydrates, highlighting the versatility of utilizing different carbohydrate sources.

Table 4.3 – Comparison for hydrogen yield of fermentation by halophilic bacteria

Carbon source	Inoculum	NaCl	T(°C)	pH	HMY	Reference
Cellobiose	*Halanaerobium hydrogeniformans*	7%	33	11	2.3±0.2 mol H_2/ mol cellobiose	(Begemann *et al.*, 2012)
Glucose	*H. hydrogeniformans*	7%	33	11	2.42 mmol H_2/ mmol glucose	(Begemann *et al.*, 2012)
Glucose	*H. salinarius*	14-15%	NI	NI	2 mM/4.17 mM	(Mouné *et al.*, 1999)
Glucose	*H. chitinovorans*	12%	37	5	144.5 µmol H_2/ 5 mL 0.5% glucose	(Liaw and Mah, 1992)
Glycerol	*H. saccharolyticum senegalensis*	150 g/L	37	7	1.6 mol H_2/ mol glycerol	(Kivistö, Santala and Karp, 2010)
Glycerol	*H. saccharolyticum saccharolyticum*	150 g/L	37	7.4	0.6 mol H_2/ mol glycerol	(Kivistö, Santala and Karp, 2010)
Glucose	Mixed culture from salt factory wastewater	75 g/L	35	8.0	0.9 ± 0.02 mol H_2/mol glucose	(Pierra *et al.*, 2013)
Glucose	Mixed culture from a salt pan	26%	35	9	1.45 mol H_2/ mol glucose	this study

T=temperature, HMY=hydrogen molar yield, NI= no information

Table 4.4 – Carbohydrate fermentation test of acclimatized seed microorganisms from Samut Sakhon salt pan compared to the pure culture of *Halanaerobium fermentans* R-9

Carbon sources	Samut sakhon acclimatized seed inoculum		*H. fermentans R-9* (Kobayashi, Kimura and Fujii, 2000)
	Fermentation test result	Biogas production (mL)	
D-glucose	+	1.27 ± 0.55	+
D-galactose	+	0.27 ± 0.06	+
D-mannose	+	1.57 ± 0.21	+
L-fucose	+	0.95 ± 0.15	NI
L-rhamnose	+	0.10 ± 0.17	-
D-xylose	+	0.30 ± 0.17	+
L-arabinose	+	1.04 ± 0.37	-
D-maltose	+	0.30 ± 0.00	+
Sucrose	+	1.00 ± 0.00	+
D-fructose	+	2.13 ± 0.32	+
D-cellobiose	+	0.27 ± 0.15	+
Trehalose	+	0.40 ± 0.17	NI
Lactose	+	0.20 ± 0.14	+
Starch	+	1.03 ± 0.06	-
α-cellulose	-	0 ± 0.00	NI
Glycerol	+	0.07 ± 0.06	-

NI: no information. Fermentation period: 6-19 days. Inoculum: 3 drops. Volume was not measured as STP

H. fermentans may have the potential to provide a valuable resource for metabolic engineering in biohydrogen production from high-salinity waste, such as pretreated lignocellulosic waste. However, genome examination will be required to identify the specific pathways that can be modified to maximize biohydrogen production yields. To date, the complete genome of this bacterium has not yet been published, unlike those of *H. saccharolyticum*, *H. praevalens*, and *H. hydrogeniformans*. The ferredoxin hydrogenase pathway is the most common hydrogen-producing exchange pathway in strict anaerobes. However, the genome of *H. hydrogeniformans* has been reported to lack this enzyme (Roush *et al.*, 2013).

Figure 4.6 – PCR-DGGE result of V3 region of the 16s rRNA from Samut Sakhon acclimatized mixed culture. The brightest bands (1 and 2) were excised, sequenced, and identified to GenBank's data base. NCBI's MegaBLAST confirmed 100% similarity of these bands (194 bp) with *H. fermentans* strain R-9.

4.4 Summary

The optimum conditions for biohydrogen production by extremely halophilic bacteria from Samut Sakhon salt pan in Thailand was determined. The highest obtained HMY was 1.45 mol H_2/mol glucose, under optimum conditions of 26% NaCl (351 g/L), 35°C, and pH 9. The acclimatized bacteria were able to ferment various types of lignocellulosic-derived sugars and other types of carbohydrates. The predominant hydrogen producer among the mixed culture was *H. fermentans*, although other unidentified bacteria also played a substantial role in the fermentation process. Future studies are needed to study biohydrogen production from lignocellulosic and food waste biomasses from the acclimatized bacteria. A new 16s rRNA sequence of *H. fermentans* strain B4 has been identified and submitted to the GenBank data base (Accession number MN133965).

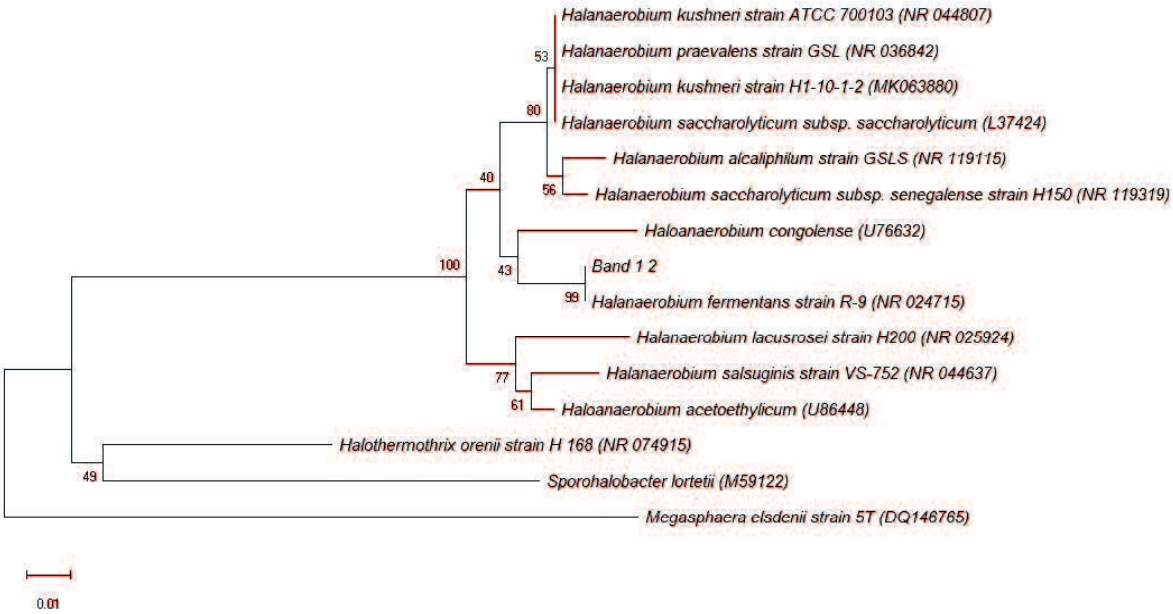

Figure 4.7 – Phylogenetic tree showing the relationships between the excised bands and other related species based on the V3 region of the 16S rRNA gene. The tree was based on Jukes-Cantor distance and constructed using the Neighbor-Joining method with 1,000 bootstraps using Mega X. The scale bar represents 0.01 substitutions per nucleotide position. *Megasphaera elsdenii* was used to root the tree. The percentage of replicate trees clustered together in the boostrap test are indicated at the nodes. Reference sequences in the dendrogram were obtained from the NCBI's GenBank.

Acknowledgements

The authors thank Yoshiki Tsuda for his valuable assistance in data collection. The authors acknowledge the technical expertise of the DNA Core facility of the Center for Gene Research, Yamaguchi University, supported by a grant-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan. The first author gratefully acknowledges the Indonesian Endowment Fund for Education (LPDP) and the Directorate General of Higher Education (DIKTI) for providing BUDI-LN scholarship. The fourth author acknowledges the Thailand Research Fund under the TRF Senior Research Scholar (RTA5980004). This work was supported by JSPS Core-to-Core Program, A. Advanced Research Networks,

which was financially supported by the Japan Society for the Promotion of Science (JSPS).

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CHAPTER V

ANALYSIS OF THE EXTREMELY HALOPHILIC BACTERIAL COMMUNITIES FROM A SALT PAN AND SALT DAMAGED SOIL IN THAILAND BY PCR-DGGE AND NEXT GENERATION SEQUENCING

5.1 Introduction

In the past 30 years, extremophilic bacteria have been gaining research interest, due to their unique adaptation strategies to survive in extreme environments. Their ability to live in severe conditions provide many advantages for constantly evolving industrial applications and genetic recombinant researches. Extremophiles consist of thermophiles, psychrophiles, barophiles, acidophiles, alkaliphiles, and halophiles. Halophiles differ from halotolerant bacteria in terms that they require NaCl for growth. Halophiles are classified by their response to NaCl, with slight halophiles has the most rapid growth at 2 to 20% (0.34-0.85 M) NaCl, moderate halophiles at 5 to 20% (0.85-3.4 M) NaCl, and extreme halophiles at 20 to 30% (3.4-5.1 M) NaCl (Larsen, 1962; Ollivier *et al.*, 1994). There have been some studies showing that higher salt concentration results in diverse volatile fatty acids and higher than usual accumulation of hydrogen (Ollivier *et al.*, 1994). However, unlike thermophilic bacteria, studies about biohydrogen production by halophilic bacteria are still very limited to date.

Biohydrogen production from lignocellulosic biomass (LCB) has been widely studied, based on the fact that LCB is steadily available as a highly generated waste in industrial processes in many countries, such as China and the United States (Sivagurunathan *et al.*, 2017). One of the main hindrances of using LCB as a feedstock to produce hydrogen is that it requires pretreatment to convert the cellulose, hemicellulose, and lignin into simple fermentable sugar forms. Among several LCB pretreatment methods that have been studied, one that has been considered the most cost-effective and widely used is alkaline pretreatment with NaOH (Kim, Lee and Kim, 2016).

One of the concerns in the biohydrogen production is the existence of methanogenic archaea that will reduce the hydrogen as fermentation product into methane. Several strategies have been taken to inhibit these methanogens in enriched mixed cultures from natural environments taken as inoculum for biohydrogen production. Those strategies include heat-shock treatment, operation at low pH, and operation at a short hydraulic retention time (Hawkes *et al.*, 2002).

Extreme halophilic bacteria have a potential role to fill several gaps in biohydrogen production from LCB. The first one is that the high salt concentration naturally inhibits the methanogens. In NaCl concentration higher than 15%, methanogenesis and sulfate reduction from hydrogen and acetate produced by fermentation has been reported to be very limited (Oren, 1988). The second one, is with their ability to grow in a high concentration of NaCl, after LCB pretreatment with NaOH and neutralization with HCl, only relatively small amount of water will be needed to dilute the pretreated biomass before fermentation process. For these reasons, the application of extremely halophilic bacteria for biohydrogen production from LCB can potentially contribute to cost reduction of sterilization and water.

With the advances of molecular biological techniques, many progress has enabled a greater understanding of the microbial diversity of hypersaline environment. Among those techniques is the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), a genetic fingerprinting technique of bacterial communities which was introduced in environmental microbiology by Muyzer et al. (Muyzer G Uitterlinden AG, de Waal EC, Uitterlinden AG *et al.*, 1993) in 1993. PCR-DGGE offers the advantage of immediate display of the genetic diversity of microbial communities. However, among the limitations of PCR-DGGE is that only major constituents in community could be identified (G. Kumar *et al.*, 2018), and it can only separate relatively short (~ 500 bp) DNA fragments (Muyzer, 1999). For the past ten years, rapid development in the Next Generation Sequencing (NGS) has made microbial community sequencing more affordable. NGS can identify minor constituents of

the microbial communities, which usually is not possible to achieve with PCR-DGGE if specific primers for the target groups were not known.

Hypersaline microbial diversities have been studied from several regions around the world. Among them were the hypersaline lakes of Dead Sea, Great Salt Lake of Utah, Lake Urmia, Iran, Siwa Oasis, Egypt, solar saltern ponds in Tunisia, Israel, Slovenia, and Dagong brine well in China (Oren, 2015; Elsaied, El-Karim and Wassel, 2017). To date, there were still limited studies about hypersaline microbial diversities from the southeast Asian regions.

This paper presents a study of three extreme halophilic microbial communities from a salt pan and salt damaged soil in Thailand, which have been screened to find potential biohydrogen producers. Both the PCR-DGGE technique and NGS with amplicon sequencing performed on IonS5™XL platform have been attempted to compare the results. The comparison will give an insight about whether PCR-DGGE technique is suitable for application in microbial communities from extreme halophilic condition.

5.2 Materials and methods

5.2.1 Inoculum and substrate

Soil for A1 cultivation bottle was taken from the shore part of salt damaged soil in Khon Kaen, while soil for A2 cultivation bottle was obtained from 10 m apart toward drier land of the salt damaged soil. Soil for B cultivation bottle was taken from the salt pan of Samut Sakhon. The substrate and method for enrichment in 26 wt% NaCl has been described elsewhere (Taroepratjeka *et al.*, 2019). Screening and enrichment have been done for three years before samples from each bottle were taken.

5.2.2 PCR-DGGE

Samples from each enrichment serum bottles were collected to have their microbial community analyzed. The genomic DNA from all of the samples were extracted and purified by using an ISOIL for Beads Beating kit (Nippon Gene Co.,

Ltd, Japan). A strategy of two-step nested PCR was implemented as an attempt to detect minor constituents of the communities.

For the first step of PCR, the 16S rRNA bacteria primers of 27F and 1492R were mixed with templates from the purified genomic DNA of each sample (1.5μL). Primer pair for the second step of nested PCR was 341F-GC and 518R for the amplification of the V3 region of the 16s rRNA. PCR products (1.5-2μL) from the first step of PCR were used as the templates for the second step. All primers were acquired from Eurofins Genomics Inc., Japan. Table 5.1 shows the oligonucleotide sequences of the primers used in this study. All PCR mixtures were prepared with EmeraldAmp PCR Master Mix (Takara Bio Inc., Japan). The methods and conditions for PCR-DGGE in this study were adapted from Kongjan et al. (Kongjan *et al.*, 2010) and Schäfer and Muyzer (Schäfer and Muyzer, 2001).

PCR products were checked using agarose electrophoresis (Mupid-2plus system, Mupid Co., Ltd. Japan) and analyzed in a ChemiDoc XRS system (Bio-Rad Laboratories, USA). For 16s rRNA PCR products with approximately 1500 base pairs (bp) length, 1% agarose (L08, Takara Bio Inc., Japan) gels were applied, while 2% agarose (S, Nippon Gene Co., Ltd., Japan) gels were used for V3 region of the 16s rRNA PCR products with approximately 200 bp length.

Polyacrylamide gels with the concentration of 8% (v/v) and 30-70% denaturant gradient were casted using Model 475 Gradient Delivery System as part of the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, USA). Denaturing solution of 100% contained 7 M urea and 40% (v/v) formamide. The amplicons of the second step of nested PCR were injected into the wells of the polyacrylamide gels with an equal volume of 2×gel loading dye (Bio-Rad Laboratories, USA). Electrophoresis was performed in 1×Tris-acetate-EDTA (TAE) buffer (Nippon Gene Co., Ltd., Japan) for 16 hours at 60°C and 70 V.

After electrophoresis, the polyacrylamide gels were stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Thermo Fisher Scientific, USA). Gels were analyzed and documented with ChemiDoc XRS system with Quantity One software (version 4.6.5; Bio-Rad Laboratories, USA). Dendrogram of the lanes was generated by FPQuest software (version 4.5; Bio-Rad Laboratories, USA) using Jaccard similarity coefficient-based UPGMA.

Excised bands were incubated overnight at 4°C in 50 µL nucleic acid-free water, and the eluates were used as DNA templates. Reamplification for each band eluate was done with two pairs of primers: the first pair with 341F-GC and 518R, the second pair with 341F (without CG clamps) and 518R, which then checked with agarose electrophoresis. PCR products with bright strips at approximately 200bp were used for the next step. The ones with the first pair of primers were inspected with DGGE, while the ones reamplified with the second pair of primers were purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co, Germany). After purification, the PCR products were sent to Yamaguchi University Center for Gene Research for Sanger sequencing. Each PCR products was sequenced with 341F and 518R primers. Using MegaX software (S. Kumar *et al.*, 2018), forward and reverse sequences of each band were aligned using MUSCLE(Edgar, 2004). The closest matches for V3 region of the 16 rRNA's sequence were searched by using MegaBLAST in GenBank data base.

Table 5.1 – Target, position, and sequences of oligonucleotide primers used for PCR

Target	**Position**	**Oligonucleotide primer sequences 5'-3'**	**References**
Bacterial 16 s rRNA gene	27F	AGA GTT TGA TCM TGG CTC AG	(Weisburg *et al.*, 1991)
(~ 1500 bp)	1495R	TAC GGY TAC CTT GTT ACG ACT T	
V3 region of the 16s rRNA gene (~200bp)	341F-GC	CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG	(Muyzer G Uitterlinden AG, de Waal EC, Uitterlinden AG *et al.*, 1993; Muyzer, 1996)
	518R	ATT ACC GCG GCT GCT GG	
	341F	CCT ACG GGA GGC AGC AG	

5.2.3 NGS

Samples from each cultivation bottles were collected and sent for high-throughput sequencing. Primers 341F and 806R were used for PCR amplifications of V3-V4 regions of the 16s rRNA. Amplicon sequencing was performed on IonS5™XL platform (Thermo Fisher Scientific, Waltham, MA, USA) at Novogene Company Limited, Hong Kong.

High quality clean reads from raw data were obtained through quality filtering of QIIME. Chimera sequences were detected using UCHIME algorithm against Gold database and removed to produce effective reads. Uparse software was then used to analyze sequences, in which sequences with 97% similarity or more were assigned to the same operational taxonomic units (OTUs). Mothur software was used against SSU rRNA database of SILVA Database at the threshold of 0.8-1 for each representative sequence for species annotation at each taxonomic rank. MUSCLE was performed to compare multiple sequences to obtain the phylogenetic relationship of all representatives of all OTUs. Species annotations were visualized with Krona (Ondov, Bergman and Phillippy, 2011). Alpha diversity indices and OTU Heatmap were calculated and generated with QIIME (Version 1.7.0). Rarefaction curve and OTUs-based Venn diagrams were displayed with R software (Version 2.15.3).

5.3 Results and discussion

5.3.1 PCR-DGGE

The PCR-DGGE microbial population profiles from the three samples shared similar band patterns, with Sample A2 showing the least similarity (69.40%) compared to the other two samples (70.83%) in a UPGMA dendrogram based on Jaccard similarity coefficient (Figure 5. 1). It was observed that soil source of Sample A2 had coarser texture compared to sample A1 and B. Coarser soils contributed to higher species richness, where larger pores increased the number of isolated water films (Chau, Bagtzoglou and Willig, 2011). From all the lanes, the bands with the highest intensity corresponded to the position of Band 5.

Theoretically, the relative intensity of the bands and their positions showed the relative abundance of each corresponding species in their populations (Muyzer G Uitterlinden AG, de Waal EC, Uitterlinden AG *et al.*, 1993).

Six bands were excised from the polyacrylamide gel as shown by the numbers in Figure 5.1, reamplified and checked with DGGE, as shown in Figure 5.2. The reamplified products turned out to be consisting of more than one bands. Most of them have bands with the highest intensity in the same position of Band 5 of the original samples. Sanger sequencing of Band 1, 2, and 5 gave results to the same sequence. MegaBLAST search of Band 5's sequence (194 bp) confirmed 100% similarity with *Halanaerobium fermentans* strain R-9.

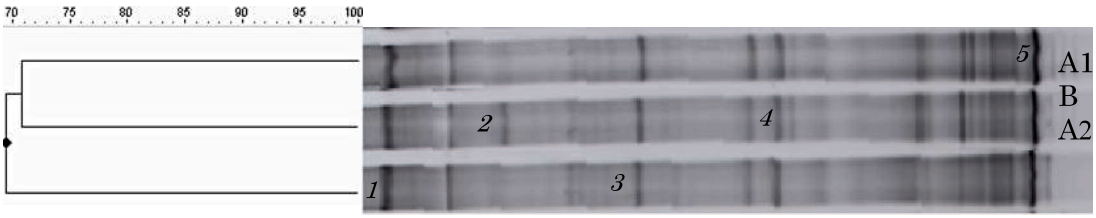

Figure 5.1 – UPGMA dendrogram generated based on the DGGE profiles of the bacterial V3 region of 16s rRNA from salt damaged soil in Khon Kaen (A1, A2) and Samut Sakhon salt pan (B). A scale of similarity ranges from 0 to 100%, where total dissimilarity is represented by 0% and completely identical is represented by 100%. The numbers in italics indicate excised bands from corresponding positions.

Several studies have also reported the same case of artifacts, or multiple bands corresponding to the same bacterium. The possible causes were heteroduplex formation in the reannealing step of PCR, preferential 16s rDNA amplification of a certain species, chimeric sequences, or the running conditions of DGGE (Hong, Pruden and Reardon, 2007; Neilson, Jordan and Maier, 2013; Subasinghe *et al.*, 2019).

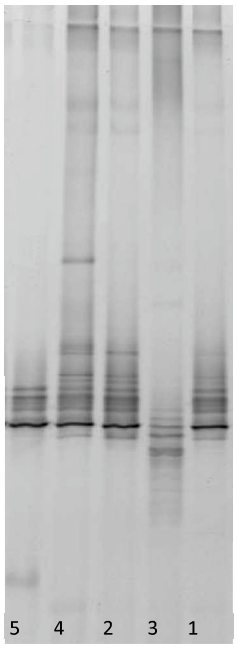

Figure 5.2 – DGGE result of the V3 region of 16s rRNA of reamplified bands which were excised from the original samples in Figure 5.1.

5.3.2 Next Generation Sequencing

A total of 355,135 sequences with an average length of 428 bp were obtained from A1, A2, and B samples. The raw sequence reads were between 95,802 and 140,177. Based on 97% threshold, a total of 345 OTUs were obtained, with a range of 76 (B) to 155 (A2) and an average of 115. The Shannon and Simpson indices (Table 5.2) shows that the microbial diversity of A3 sample was much higher than the other two samples, with A1 sample being the lowest, although not so much different than B sample. Chao richness estimator also indicates A2 sample having the most of rare OTUs, followed by A1 and B sample. The same trend also applies to the ACE method, which estimates the number of species through sample coverage (Kim *et al.*, 2017).

Good's coverage, which estimates sampling completeness by calculating the probability of randomly selected sequences of the amplicons (Nam, Lee and Lim, 2012), shows the score of 1 for all the samples, suggesting that all of the bacterial

community in the samples has been captured at the current sequential depth. Phylogenetic diversity (PD) whole tree, provides a measure of diversity based on the quantification of phylogenetic tree's branch diversity (Pylro *et al.*, 2014). The PD whole tree scores were not so different for A2 and A1, but sample B showed much lower diversity between all the samples.

Rarefaction curves provide a way to compare observed richness from communities which have been sampled unequally, by measuring observed OTUs at a certain depth of sequencing (Kim *et al.*, 2017). Sample A2 showed the highest observed species number, followed by samples A1 and B (Figure 5.3). Other than 55 OTUs which were commonly shared by the three samples, Sample A2 also has 61 OTUs which were unique to the sample, compared to 17 OTUs of A1 and 5 OTUs of B (Figure 5.4).

Table 5.2 – Alpha diversity indices

Sample name	Observed species	Shannon	Simpson	Chao1	ACE	Good's coverage	PD whole tree
A1	109	0.777	0.189	118.231	117.204	1.000	13.401
A2	155	3.152	0.791	155.652	156.894	1.000	14.512
B	68	0.904	0.228	99.667	96.987	1.000	6.243

MegaBLAST analysis was performed for six most abundant OTUs (Figure 5.5 and Table 5.3). OTU_128 was the most abundant for Sample A1 and B. Even though the other five OTUs appeared abundantly in Sample A2, they're only available in small counts in the other two samples. While OTU_128 shares a high similarity with *Halanerobium fermentans* R-9, OTU_94, OTU_40, OTU_49, OTU_158, and OTU_96 all show similarities with the same species, *Halanaerobacter lacunarum* TB21.

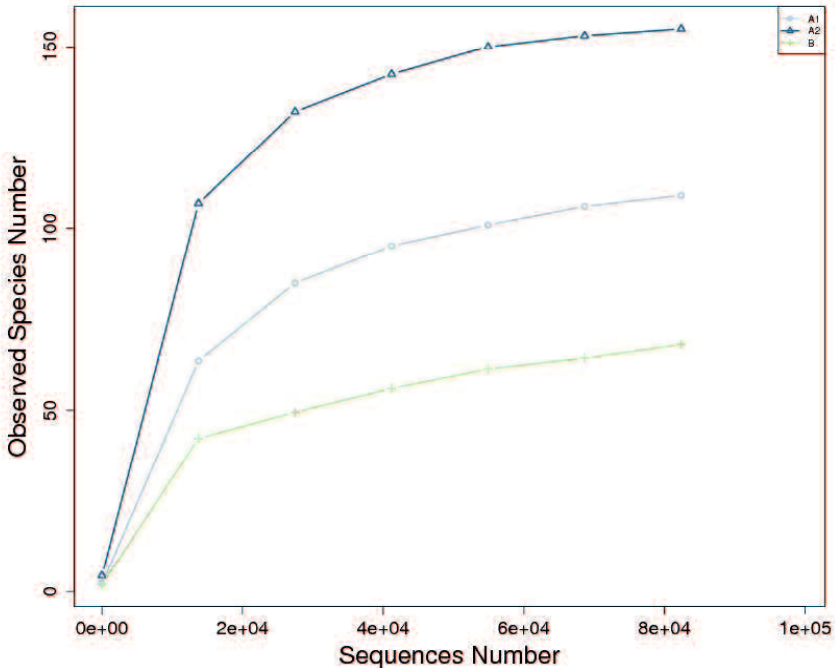

Figure 5.3 – Rarefaction curves of sample A1, A2, and B (△ =A2, ○ = A1, + = B)

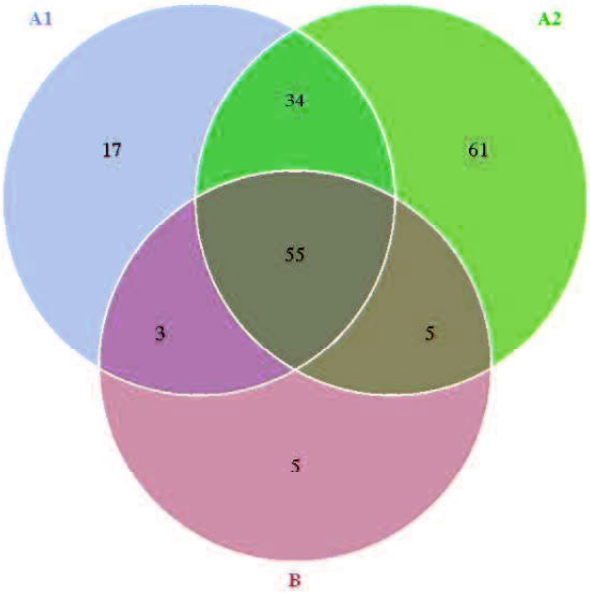

Figure 5.4 – Venn diagram based on OTUs. Each circle represents one sample. Values in overlapping parts represent common OTUs.

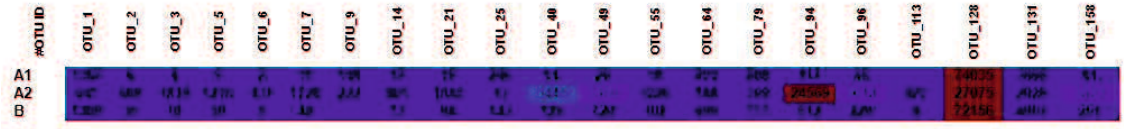

Figure 5.5 – OTU heat-map (filter by counts per OTU:300)

H. fermentans (basonym: *Haloanaerobium fermentans*) R-9, was reported to grow in 7% to 25% NaCl, with the optimum NaCl concentration of 10% (Kobayashi, Kimura and Fujii, 2000). The temperature range for *H. fermentans* is between 15 to 45°C, with the optimum temperature of 35°C. It can live in pH 6 to 9, with the optimum pH of 7.5. The species was isolated for the first time from Japanese fermented puffer fish ovaries, and able to ferment glucose, cellobiose, fructose, galactose, lactose, maltose, mannose, raffinose, ribose, sucrose, and xylose (Kobayashi, Kimura and Fujii, 2000).

Table 5.3 – Phylogenetic identification results of the most abundant OTUs

OTU number	Sequence length (bp)	Closest relative (NCBI accesion no.)	Similarity
OTU_128	448	*Halanaerobium fermentans* R-9 (NR 024715.1)	99.55%
OTU_94	447	*Halanaerobacter lacunarum* TB21 (KJ677978.1)	97.45%
OTU_40	449	*Halanaerobacter lacunarum* TB21 (KJ677978.1)	97.51%
OTU_49	450	*Halanaerobacter lacunarum* TB21 (KJ677978.1)	99.03%
OTU_158	438	*Halanaerobacter lacunarum* TB21 (KJ677978.1)	96.79%
OTU_96	438	*Halanaerobacter lacunarum* TB21 (KJ677978.1)	96.80%

The extremely halophilic *H. lacunarum* (basonym: *Halobacteroides lacunaris*) TB21, was isolated from deep-sea hypersaline anoxic brine *Thetis* in the Eastern Mediterranean Sea (Lorenzo *et al.*, 2017). It is an obligate halophilic which grows in NaCl concentration between 1.7 M and 5.5 M (10%-32%), with the optimum NaCl of 15-15% (Mouné *et al.*, 1999; Lorenzo *et al.*, 2017). *H. lacuranum* can live in temperature between 25 to 52°C, with the optimum range of 35 to 40 °C. The pH range is between 6.5 to 8, with the optimum pH of 6.5 to 7 (Mouné *et al.*, 1999; Mezghani *et al.*, 2012).

Krona analysis of the species annotation (Figure 5.6) shows the most abundant genus for each sample. A1 and B each have 98% and 97% of *Halanaerobium* in them, while A2 is dominated by *Halanaerobacter* (61%), followed by *Halanaerobium* (37%).

5.3.3 Comparison between PCR-DGGE and NGS results and biohydrogen production

The higher population of *Halanaerobacter* genus instead of *Halanaerobium* in Sample A2 (Figure 6) was unexpected since the band intensities and patterns of the DGGE image of the respective sample doesn't differ much with the other two. Perhaps this was due to the similar DNA G+C contents of most abundant species related to the genus, which was 32.4 mol% for *H. lacunarum* and 33 mol% for *H. fermentans* (Kobayashi, Kimura and Fujii, 2000; Mezghani *et al.*, 2012). One study found that for some communities, DGGE bands of the same horizontal positions do not always represent the same organism (Hong, Pruden and Reardon, 2007). Moreover, Muyzer also stated that separations of fragments having different sequences sometimes are not possible due to the fragments' similar melting behavior (Muyzer, 1999).

In terms of biohydrogen production, higher diversity does not necessarily give a better result. From a previous study, it was found that Sample A2 produced the lowest hydrogen molar yield (HMY) of 0.66 mol H_2/mol glucose, compared to

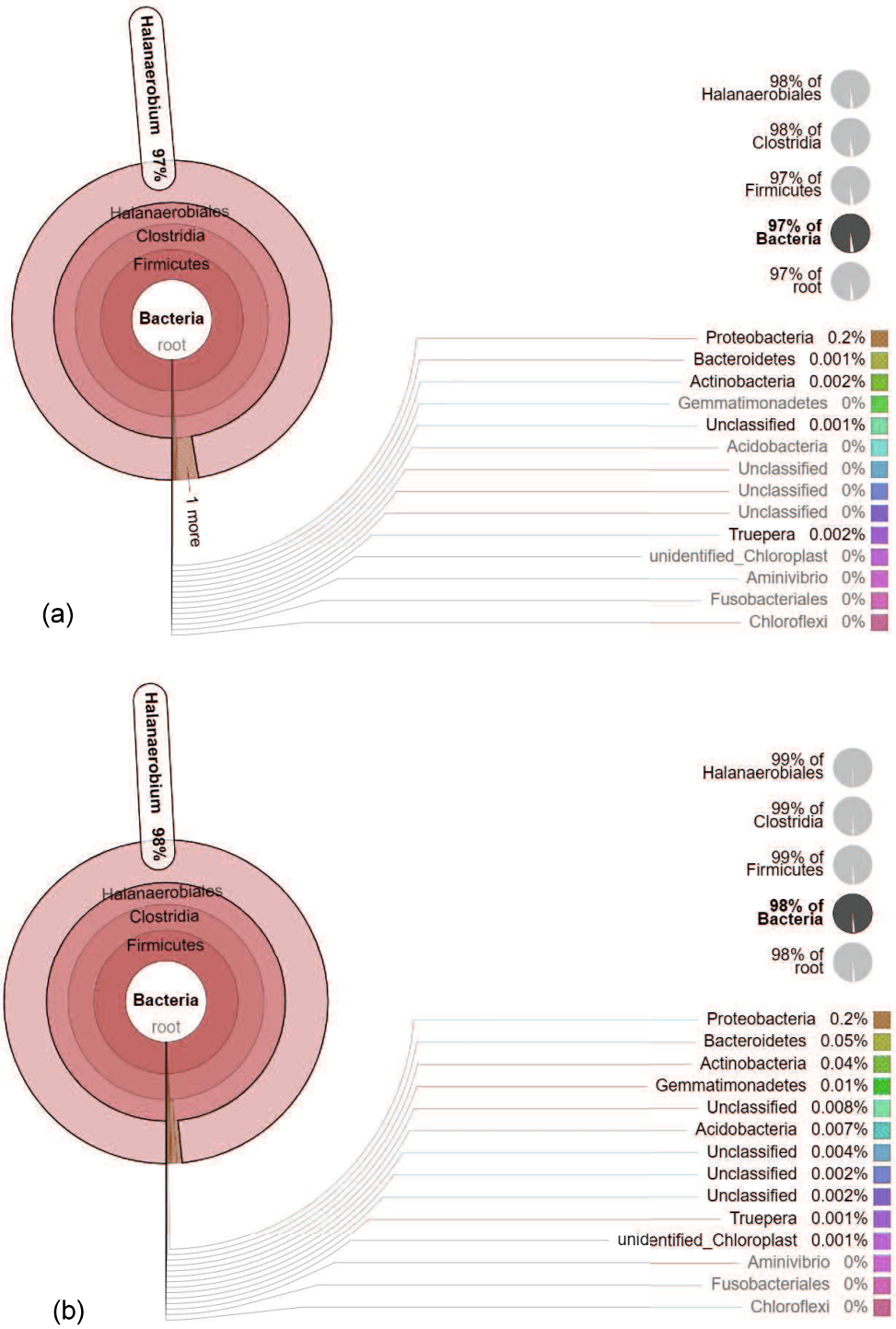

Figure 5.6 (a) and (b) – KRONA analysis result of species annotation showing the most abundant genus of sample A1 and B

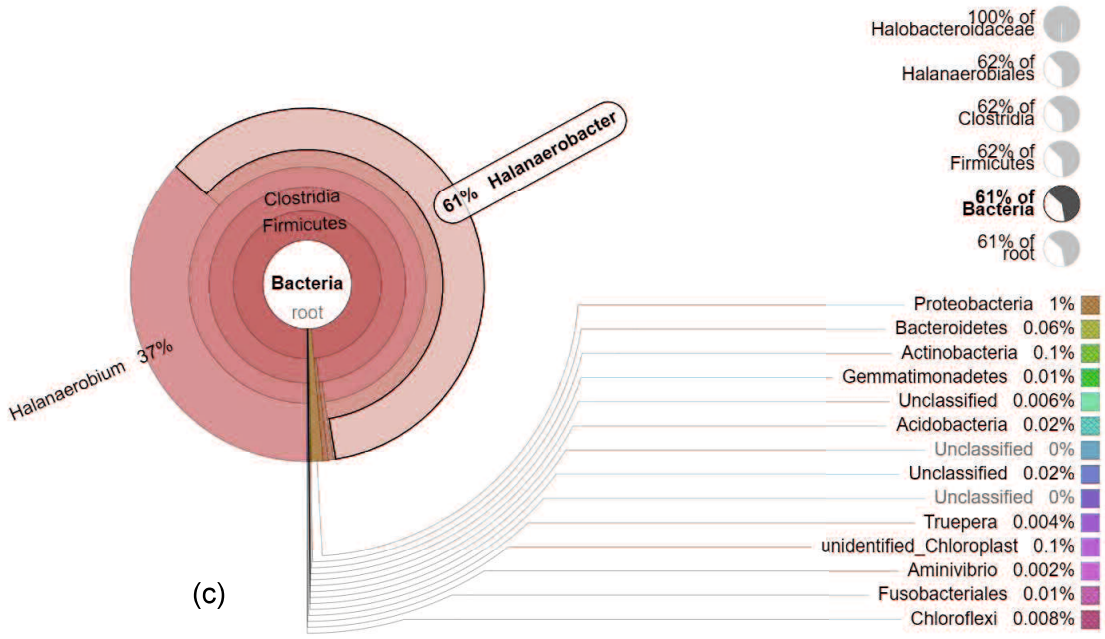

Figure 5.6 (c) – KRONA analysis result of species annotation showing the most abundant genus of sample B, and A2

Sample B which produced 1.08 mol H_2/mol glucose and Sample A1 with 1.15 mol H_2/mol glucose in temperature of 37°C and unadjusted initial pH conditions (Taroepratjeka *et al.*, 2019).This lower yield might be related to the different abilities of *H. lacunarum* and *H. fermentans* in producing biohydrogen, but a further study should be carried out for confirmation.

Through dark fermentation, hydrogen is usually produced through the the acetic acid or butyric acid pathways. Theoretically, from each gram of glucose in standard temperature and pressure condition, the acetic acid pathway produces 498 mL of hydrogen, while the butyric acid pathway produces 249 mL of hydrogen (Taroepratjeka *et al.*, 2019). Both *H. fermentans* and *H. lacunarum* are able to produce acetate, H_2, CO_2, and ethanol, with the addition of formate and lactate for *H. fermentans* (Mouné *et al.*, 1999; Kobayashi, Kimura and Fujii, 2000). Butyrate has not been reported as a fermentation product for both species.

5.3.4 Comparison between NGS results and carbohydrate fermentation test

Table 4.4 shows a comparison of carbohydrate fermentation test's results between the acclimatized seed microorganisms from Sample B and the pure culture of *H. fermentans* strain R-9. The difference in carbohydrate utilization ability was observed for L-arabinose, L-rhamnose, starch, and glycerol, which were fermentable by the mixed culture, but showed negative results in the report by Kobayashi *et al.* (Kobayashi, Kimura and Fujii, 2000).

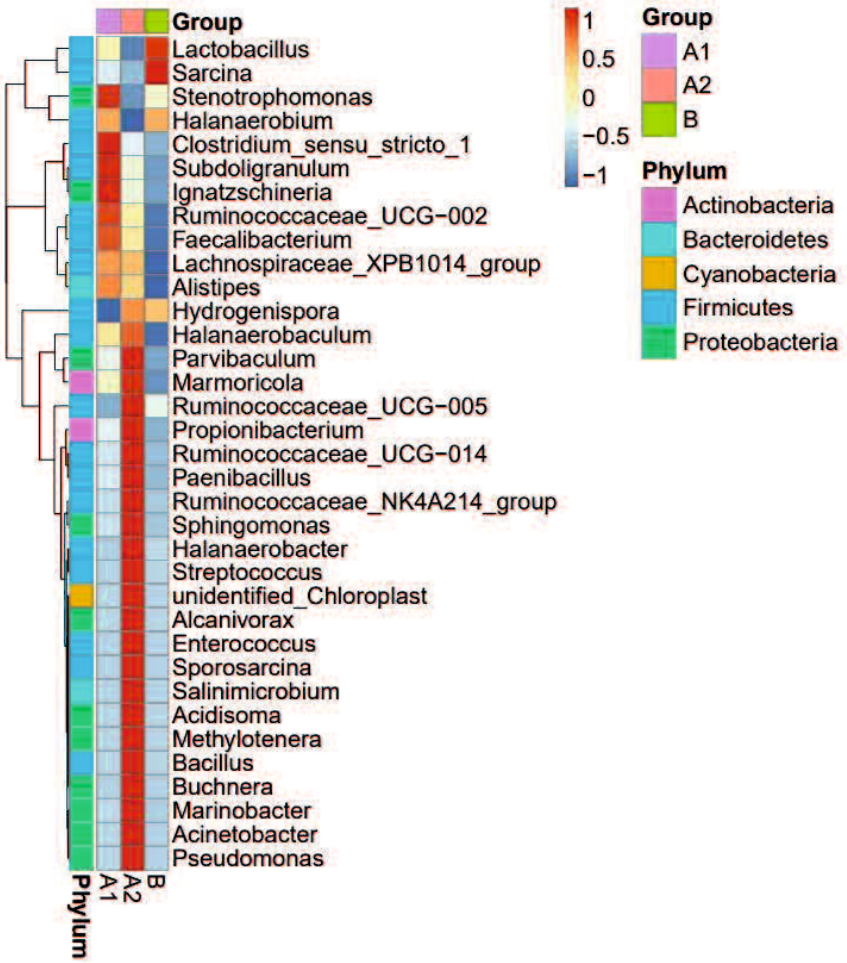

Figure 5.7– Species abundance heatmap among the samples

The species abundance heatmap among the samples (Figure 5.7) showed that other fermenting genus such as *Lactobacillus sp.*, *Sarcina sp.* and

Hydrogenispora sp were also present in Sample B, with the abundance analyzed by Krona of 0.2%, 0.008%, and 0.01%, respectively.

Lactobacillus genus consist of 180 species which primarily ferment sugars to lactic acid. Among the two species that belongs to *Sarcina* genus, *Sarcina ventriculi* has been reported to ferment up to 20 g/L of arabinose, with the main products of ethanol, acetate, CO_2, and H_2 (Finn, Bringer and Sahm, 1984). Only one species belongs to *Hydrogenispora* genus, which is *Hydrogenispora ethanolica*. This bacteria was able to fermentatively grow on arabinose, starch, and glycerol besides glucose, maltose, fructose, ribose, xylose, galactose, raffinose, mannose, pectin, sucrose, fumarate, yeast extract, and tryptone (Liu *et al.*, 2014).

5.4 Summary

In this study, PCR-DGGE was only able to provide a rough idea of the extremely halophilic bacterial communities cultivated from the soils of Samut Sakhon salt pan and salt damaged soil in Khon Kaen, Thailand. Amplicon sequencing with NGS provided more detailed pictures of those communities and explained why several bands in DGGE corresponded to the same genus. A metagenomic study could contribute to obtaining a clearer picture of the genes and metabolic pathways available in the communities. These communities can be potential resources for biohydrogen production in extremely halophilic condition.

Acknowledgements

The authors acknowledge the technical expertise of the DNA Core facility of the Center for Gene Research, Yamaguchi University, supported by a grant-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan. The first author gratefully acknowledges the Directorate General of Higher Education (DIKTI) and the Indonesian Endowment Fund for Education (LPDP) for providing BUDI-LN scholarship. The fourth author acknowledges the Thailand Research Fund under the TRF Senior Research Scholar (RTA5980004). This work was supported by JSPS Core-to-Core Program, A. Advanced Research Networks,

which was financially supported by the Japan Society for the Promotion of Science (JSPS).

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CHAPTER VI

CONCLUSIONS, RESEARCH LIMITATIONS, AND FUTURE WORKS

6.1 Conclusions

Below are the conclusions gathered from this study:

- The experimental results showed that it is possible to produce biohydrogen under high salt concentrations (26% NaCl) after at least one year of acclimatization.
- The optimum conditions for biohydrogen production by the extremely halophilic bacteria community from Samut Sakhon salt pan in Thailand was determined. The highest obtained HMY was 1.45 mol H_2/mol glucose, under optimum conditions of 26% NaCl (351 g/L), 35°C, and pH 9.
- After identification with PCR-DGGE and Next Generation Sequencing, predominant hydrogen producer among the acclimatized bacterial communities was found to be *Halanaerobium fermentans*.
- A new 16s rRNA sequence of *H. fermentans* strain B4 has been identified and submitted to the GenBank data base (Accession number MN133965). The 1,424-base pair length sequence shares 99.36% similarity with strain R-9, which has been isolated from salted puffer fish ovaries in Japan and reported in 2000.
- The acclimatized bacterial community from Samut Sakhon was able to degrade carbohydrates unfermentable by pure culture of *H. fermentans* due to the small presence of *Lactobacillus sp*. (0.2%), *Sarcina sp*. (0.008%), and *Hydrogenispora sp*. (0.01%).

6.2 Research limitations

Although dark fermentation with extremely halophilic bacteria could offer an affordable process for hydrogen production, the pretreatment cost of the

lignocellulosic biomass remains a challenge until now. Due to the scope and time constraints, this study only focused on the dark fermentation process and in-depth analysis of the microbial communities of the salt pan and salt damaged soil and did not cover the part of lignocellulosic biomass pretreatment process. Figure 6.1 shows all the process involved for biohydrogen production from lignocellulosic biomass. A technology roadmap for biohydrogen production from lignocellulosic biomass can be found in Appendix B.

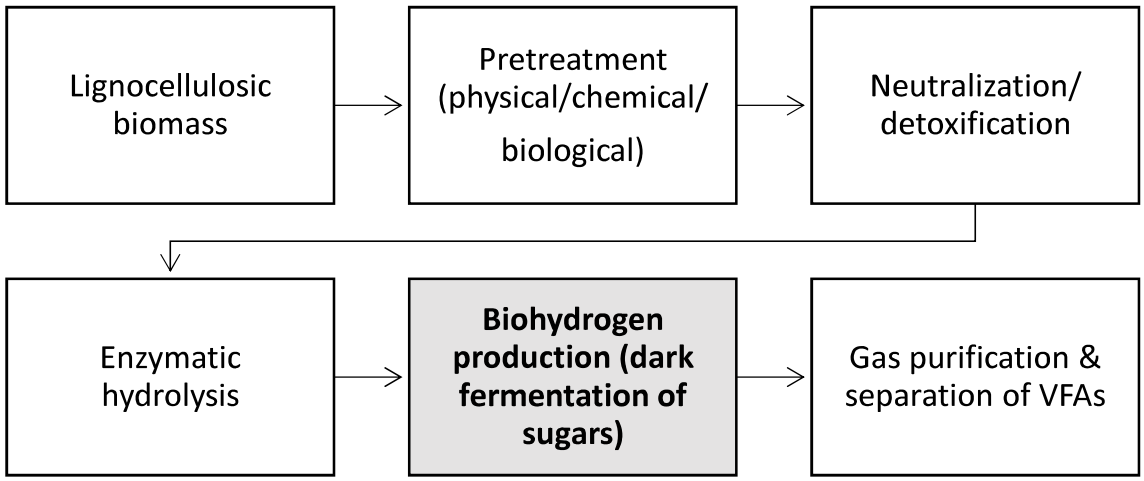


Figure 6.1 – Processes in biohydrogen production from lignocellulosic biomass. The box in grey and bold text shows the part covered in this study

6.3 Future works

Suggestions for future research topics concerning the acclimatized bacterial communities are listed below:

- Optimization of substrate to increase the hydrogen molar yield.
- A repeat experiment of the bacteria's requirement for chloride ions in high salinity condition with only Na_2SO_4 salt to confirm the results.

- Optimization of salinity and temperature for shorter hydraulic retention time to cut operating cost in reactors.
- A study of biohydrogen production from lignocellulosic and food waste biomasses by the acclimatized bacteria through anaerobic liquid state and solid-state fermentation.
- A metagenomic study to obtain a clearer picture of the genes and metabolic pathways available in the communities.

APPENDIX A

LIST OF PUBLICATIONS

1. **Taroepratjeka D.**, Imai T., Chairattanamanokorn P., Reungsang A. Biohydrogen production by extremely halophilic bacteria from the salt pan of Samut Sakhon, Thailand. *Chiang Mai Journal of Science*. (Accepted on October 8, 2019).
2. **Taroepratjeka D.**, Imai T., Chairattanamanokorn P., Reungsang A. Investigation of hydrogen-producing ability of extremely halotolerant bacteria from a salt pan and salt-damaged soil in Thailand. *International Journal of Hydrogen Energy*, **44**(6), 3407-3413.

LIST OF PRESENTATIONS

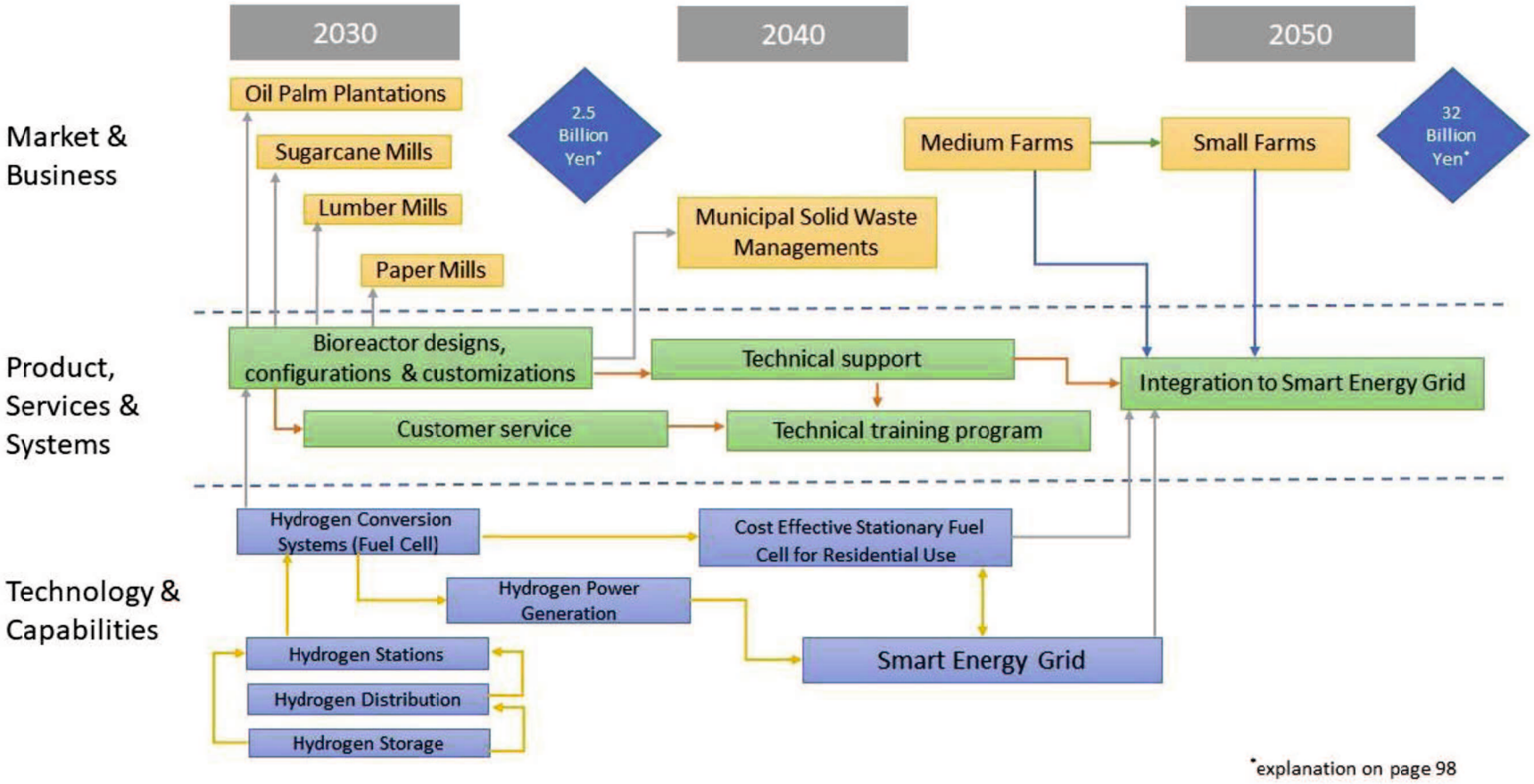
1. Imai T., **Taroepratjeka D.**, Chairattanamanokorn P., Reungsang A. Microbial community analysis of extremely halophilic bacteria from a salt pan and salt damages soil in Thailand for biohydrogen production from lignocellulosic biomass. *The 12th International Conference on the Challenges in Environmental Science and Engineering.* Kaohsiung, Taiwan. November 3-7, 2019. (Oral presentation)
2. **Imai T.**, Taroepratjeka D. Screening and identification of extremely halophilic bacteria for biohydrogen production from lignocellulosic biomass. *10th IWA International Symposium on Waste Management Problems in Agro-Industries* (*Agro'2019)*. Rhodes Island, Greece. June 19-21, 2019. (Oral presentation)
3. **Taroepratjeka D.**, Imai T., Chairattanamanokorn P., Reungsang A. Biohydrogen production by halophilic bacteria from Samut Sakhon Salt Pan in Thailand. *The 5th International Symposium "Green and Smart Technologies for a Sustainable Society"*. Faculty of Engineering, Yamaguchi University. March 25-27, 2019. (Poster presentation)
4. **Taroepratjeka D.** & Imai T. Microbial community characteristics of halotolerant hydrogen producing bacteria from salt pan and salt damages

soil in Thailand. *15th Young Scientist Seminar "Establishment of International Research Network for Tropical Bioresources and Their Utilization"*. Yamaguchi Prefectural Seminar Park, Japan. November 13-14, 2017. (Oral presentation)

5. **Taroepratjeka D.**, Imai T., Chairattanamanokorn P., Reungsang A. Hydrogen producing ability of extremely halotolerant bacteria from salt-damaged soil in Thailand. *Water and Environment Technology Conference 2018*. Ehime University, Japan. July 14-15, 2018. (Oral and poster presentation)
6. **Taroepratjeka D.** & Imai T. Bio-hydrogen production from lignocellulosic biomass by extremely salt tolerant bacteria: effect of initial pH on H_2 production. *13th Young Scientist Seminar "Establishment of International Research Network for Tropical Bioresources and Their Utilization"*. Yamaguchi Prefectural Seminar Park, Japan. November 18-19, 2017. (Oral presentation)
7. **Taroepratjeka D.**, Syafila M., Imai T. Kinetics of aerobic sequencing batch reactor after dissolved air flotation for slaughterhouse wastewater treatment in Bandung, Indonesia. *Water and Environment Technology Conference 2017*. Hokkaido University, Japan. July 22-23, 2017. (Oral and poster presentation)

APPENDIX B

BIOHYDROGEN PRODUCTION FROM LIGNOCELLULOSIC BIOMASS TECHNOLOGY ROADMAP

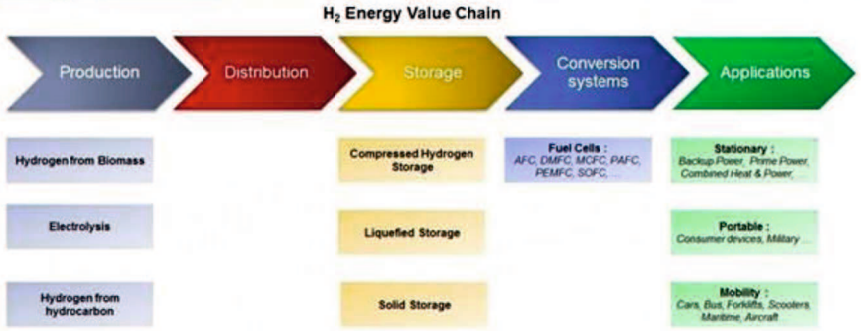


Linkage Grids

- Objective: To sell customized in situ bioreactor for biohydrogen production from cellulosic biomass waste.
- Milestone 1 (2035): 0.25% of 1 trillion yen (please see "Strategic Road Map for Hydrogen and Fuel Cells" on page 3).
- Milestone 2 (2050): 0.4% of 8 trillion yen (please see "Strategic Road Map for Hydrogen and Fuel Cells" on page 3).

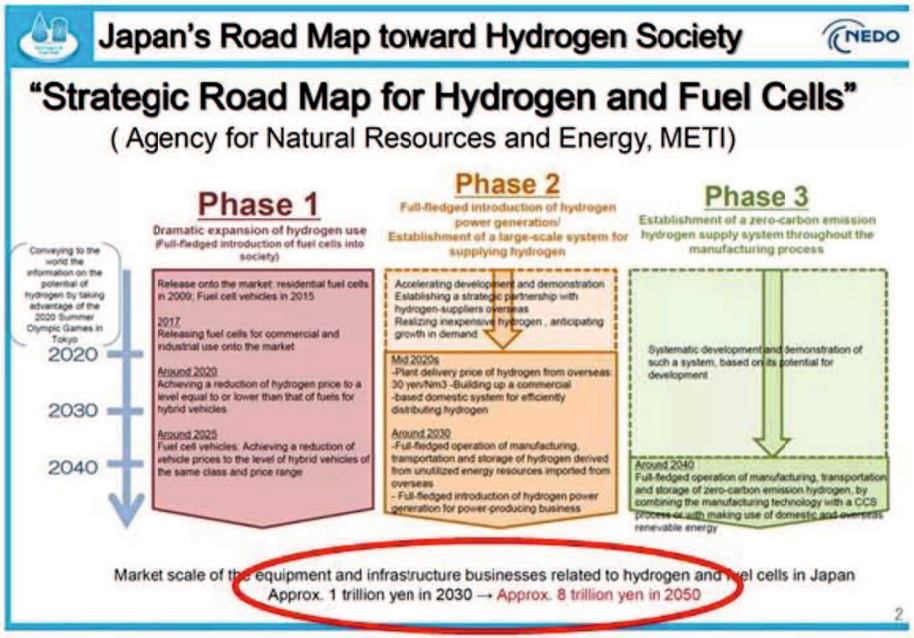
	Product, Service & System						
Technology & Capabilities		Bioreactor designs, configurations & customizations	Customer service	Technical support	Technical training program	Integration to Smart Energy Grid	
	Cost Effective Stationary Fuel Cell for Residential Use	✓✓✓	✓✓	✓✓	✓✓✓	✓✓✓	13
	Hydrogen Power Generation	✓✓✓	✓	✓		✓✓✓	8
	Hydrogen Stations	✓✓	✓	✓		✓	7
	Smart Energy Grid		✓	✓✓	✓	✓✓✓	7
	Hydrogen Conversion Systems (Fuel Cell)	✓	✓	✓		✓✓✓	6
	Hydrogen Storage	✓✓✓	✓	✓		✓	6
	Hydrogen Distribution	✓✓✓				✓	4

	Market & Business							
Product, Service & System		Oil Palm Plantation	Sugarcane Mills	Lumber Mills	Paper Mills	Municipal Solid Waste Managements	Medium Farms	Small Farms	
	Bioreactor designs, configurations & customizations	✓✓✓	✓✓✓	✓✓✓	✓✓✓	✓✓✓	✓✓✓	✓✓✓	21
	Technical support	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓✓	15
	Customer service	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	✓✓	14
	Technical training program	✓✓	✓✓	✓✓	✓✓	✓✓	✓	✓	12
	Integration to Smart Energy Grid	✓	✓	✓	✓	✓	✓✓✓	✓✓✓	11



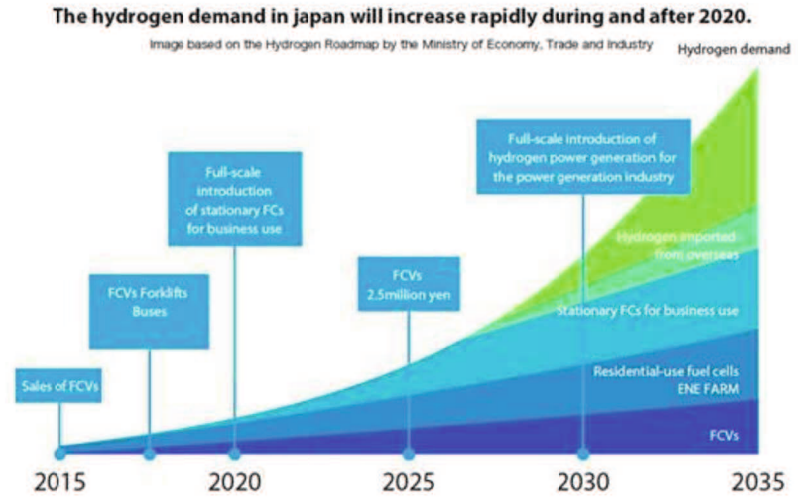
Taken from http://www.infinergia.com/en/fuelcell%20

Data Sources for Roadmap



Taken from http://www.ammoniaenergy.org/on-the-ground-in-japan-residential-fuel-cells/
Source: "NEDO's R&D program for Hydrogen and Fuel Cell Towards Hydrogen Society." Yoshihiro Shinka, New Energy and Industrial Technology Development Organization (NEDO). November 30, 2015.

Other references:
http://www.meti.go.jp/english/press/2014/pdf/0624_04a.pdf
https://www.iea.org/publications/freepublications/publication/smartgrids_roadmap.pdf



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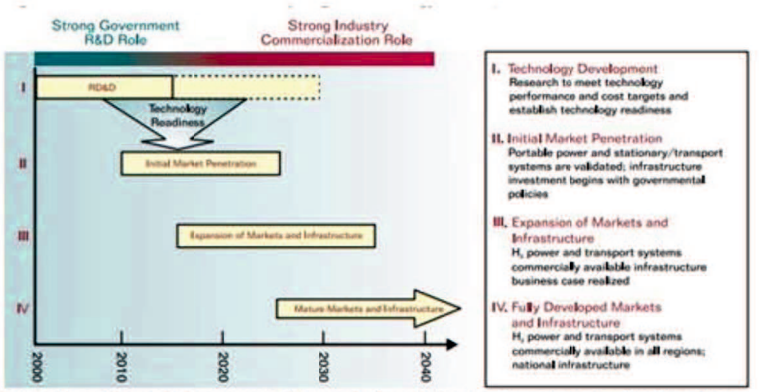

The timeframe is long and the investment is large to develop a hydrogen and transportation market that reduces our Nation's dependence on foreign sources of energy while minimizing environmental impacts.

Taken from "Hydrogen Posture Plan: An Integrated Integrated Research, Development and Demonstration Plan," United States Department of Energy & United States Department of Transportation, December 2006
http://large.stanford.edu/courses/2015/ph240/llanos1/docs/posture-2006.pdf