博士論文
Doctoral Dissertation

DEVELOPMENT OF BIO-HYDROGEN PRODUCTION PROCESS FROM WOOD MATERIAL AS A RENEWABLE FEEDSTOCK

(木質系バイオマスからのバイオ水素生産プロセスの開発)

２０１５年３月

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ABSTRACT

Fossil fuel energy crisis and awareness of climate change are concerned recently; the development of clean alternative energy sources is now significantly interested worldwide. Bio-hydrogen (H_2) produced from lignocellulosic biomass is one of promising candidate due to its clean, CO_2-free, and high efficiency energy carrier to meet the future needs. However, lignocellulosic materials require pretreatment and hydrolysis prior using as a feedstock in dark fermentation.

The waste from disposable wooden chopsticks (DWC) was represented as wood waste and investigated in this study. A thermophilic anaerobic mixed culture obtained from a hot spring was used as inoculum for fermentative H_2 production, and its optimum initial pH and temperature were determined to be 7.0 and 50°C, respectively. During the fermentation, acetic and butyric acids were also produced along with H_2 production, thus it could be concluded that acetate-butyrate fermentation occurred during H_2 production by mixed culture used in this study. Moreover, microbial community of the thermophilic anaerobic mixed culture had been characterized, *Thermoanaerobacterium* spp. were the H_2-producing bacteria mainly present in the fermentation process.

The feasibility of using DWC as a feedstock for fermentative H_2 production was established in this study. H_2 production from DWC may be enhanced by removing lignin and increasing the porosity of the material prior to enzymatic hydrolysis. Alkaline pretreatment, used to delignify DWC waste, were investigated in this study. The effects of NaOH concentration, temperature and retention time were examined and it was found that pretreatment time had no effect on lignin removal or carbohydrate released in enzymatic hydrolysate. The highest percentage of lignin removal (41%) was obtained with 2% NaOH at 100°C, correlated with the highest sugar released (67 mg/g pretreated DWC) in the enzymatic hydrolysate. Moreover, the surface structure of the pretreated DWC was changed from smooth to rough in comparison to raw DWC. These evidences supported that alkaline pretreatment degraded lignin mainly from the cellulosic biomass, and this allowed enzyme to obtain easier access to cellulose molecules during enzymatic hydrolysis. Most of inhibitors (phenolic compounds) were released into the pretreatment liquid phase, so that the total phenolics content in the enzyme hydrolysate was much lower. Thus, detoxification was not required after pretreatment; hydrolysate could be used directly as a feedstock for H_2 production in this study. The enzymatic hydrolysate was then used as substrate for H_2

production by anaerobic mixed culture enriched from hot spring and yielded 8 mL H_2/g pretreated DWC (6.4 mL H_2/g DWC).

According to H_2 production yield was low; it might due to the fact that low sugar concentration was obtained in enzymatic hydrolysate. So that the enzymatic hydrolysis step needed to be improved the efficiency by optimizing its condition. In order to optimize the condition of the enzymatic hydrolysis of alkaline pretreated DWC, response surface methodology is an efficient experimental tool used to determine the optimal conditions of numerous variables. In the present study, cellulase dosage, β-glucosidase dosage, Tween 80, and hydrolysis time, were found to have a significant effect on enzymatic hydrolysis based on the Plackett-Burman design (PBD). Those factors were subsequently investigated on the optimal levels by a central composite design (CCD), which was determined at 36 FPU/g pretreated DWC of cellulase, 53 CBU/g pretreated DWC of β-glucosidase, 0.4 g/g pretreated DWC of Tween 80 for 105 h. Under optimal conditions, glucose and reducing sugar yielded at 121.7 and 435.8 mg/g pretreated DWC, respectively. Reducing sugar yield was increased 4.6 fold after optimization of enzymatic hydrolysis condition.

In order to enhance H_2 production from enzymatic hydrolysate by a thermophilic anaerobic mixed culture, optimization of a medium composition was employed in this section. Seven medium components were screened using PBD, three out of seven components (yeast extract, phosphate buffer, and $FeSO_4 \cdot 7H_2O$) were found to have a significant effect on H_2 production. These components subsequently optimized their concentration via response surface methodology using CCD. The maximum H_2 production was achieved at 1,949 mL H_2/L pretreated DWC hydrolysate under optimum conditions using 6.74 g/L yeast extract, 29.62 mM phosphate buffer and 0.05 g/L $FeSO_4 \cdot 7H_2O$. In addition, the results found that fermentation pathway was possibly shifted from acetate to acetate-ethanol type fermentation when the concentration of yeast extract was increased together with increasing the concentration of phosphate buffer. H_2 production was yield approximately 40 mL H_2/g pretreated DWC (32 mL H_2/g DWC). The results in this study show a potential application using DWC as a renewable feedstock for fermentative H_2 production. Furthermore, this process could be applied on other hardwood waste i.e. wood chips, bark form forest residue; saw dust from furniture factory and wooden waste from demolishing construction, etc. Even though, the cost operation was considered and found relatively high in this study since the initial phase of development of H_2 production process from wood material was just

investigated by this work. However, there are a feasibility to be improved the fermentative H_2 production process by several aspects and efforts in order to be more applicable in practice.

学位論文内容の要旨

近年，化石燃料の枯渇ならびにその使用にともなう二酸化炭素排出によって地球温暖化が大きな地球規模の問題となっており，それゆえにクリーンエネルギーである水素に注目が集まっている．水素の生産方法としては種々あるが，近年注目されているのはバイオマスを原料とした生物学的水素生産である．水素はエネルギーとして使用する際には水しか排出しないものの，その生産時には必然的に二酸化炭素が副生する．しかしながら，水素生産にバイオマスを原料とすることで，その副生する二酸化炭素さえもカーボンニュートラルとすることができ，極めて持続可能的にエネルギーを得ることができる．様々なバイオマスの中で，最も利用が進んでいないのが木質系バイオマスであり，本研究ではそれ（本研究では割り箸を対象とした）を対象に水素生産プロセスの開発を行った．

水素生産の方法としては嫌気性微生物による生物学的高温水素発酵を用いた．まず，高温水素発酵に適する微生物を大分県別府温泉の温泉湧出口付近の底泥からスクリーニングし，その最適水素発酵条件を求めた．実験結果から，最適条件は初期 pH が 7.0 で，培養温度が 50℃であることが明らかとなった．水素発酵過程で酢酸及び酪酸が副生したことから，酢酸ー酪酸経路の水素発酵であることがわかった．また，優占する水素発酵細菌は *Thermoanaerobacterium* spp.であることが分子生物学的手法により明らかとなった．

次に，割り箸はそのままでは水素発酵できず，最初にリグニンを分解して空隙を増加させてから，その加水分解物の酵素処理により糖化を行う必要がある．したがって，アルカリ処理によりリグニンを分解する条件の把握を行った．アルカリの濃度，処理温度，処理時間について検討を行った結果，処理の時間はほとんど影響しないことがわかった．最大のリグニン除去率（41%），それに続く加水分解物の酵素処理による最大の糖化量（67 mg/g pretreated DWC）が 2%の NaOH，100℃の処理温度の場合に得られた．さらに，割り箸の表面は処理前に比べ処理後は明らかに粗くなっていたことが観察された．これらのことから，アルカリ処理によりリグニンを分解し，その後の加水分解物の酵素処理による糖化量を向上できることが明らかとなった．加水分解物の酵素処理による糖化時に阻害を及ぼす阻害性物質であるフェノール化合物は前処理の液相にそのほとんどが移行し，それゆえに加水分解物

の酵素処理による糖化にほとんど影響しないことが明らかとなった．すなわち，加水分解物から阻害物質を除く工程が必要なく，アルカリによる前処理後にそのまま加水分解物を水素発酵に供することができる．上記の加水分解物を用いて，水素発酵を行ったところ 8 mL-H_2/g-pretreated -DWC（廃棄割り箸）という結果となった．この結果はまだ低く，十分な水素収率が得られていないことがわかった．

そこで，酵素による加水分解（糖化）を一層向上させるために，その最適条件の検討を統計学的解析手法（RSM 法）により行った．Plackett-Burman Design（PBD）による解析結果から酵素（セルラーゼ）の添加量，β-グルコシダーゼの添加量，界面活性剤（Tween 80），処理時間が加水分解に影響を与える因子であることがわかった．引き続き，これらの因子の最適値を Central Composite Design（CCD）により求めた．すなわち，酵素（セルラーゼ）の添加量が 36 FPU/g pretreated DWC，β-グルコシダーゼの添加量が 53 CBU/g pretreated DWC，界面活性剤（Tween 80）が 0.4 g/g pretreated DWC，処理時間が 105 時間であった．この条件下で，グルコースと還元糖の収率はそれぞれ 121.7, 435.8 mg/g pretreated DWC であり，還元糖の収率は 4.6 倍向上した．

続いて，高温嫌気性菌を用いて上記の加水分解物からの水素生産を向上させるため，培地の組成を最適化した．Plackett-Burman Design（PBD）による解析結果から，7 つの培地組成から 3 つ（酵母エキス，リン酸バッファー，硫酸鉄）が水素生産に特に影響を及ぼすものとして選択された．引き続き，これらの最適値を Central Composite Design（CCD）により求めた．すなわち，酵母エキスが 6.74 g/L，リン酸バッファーが 29.62 mM，硫酸鉄が 0.05 g/L であった．この条件下で，水素生産の最大値は 1,949 mL H_2/L pretreated DWC hydrolysate が得られた．加えて，酵母エキスとリン酸バッファーの添加量が双方とも増加した場合に，水素発酵の経路が酢酸タイプから酢酸ーエタノールタイプにシフトしたと予測された．水素収率としては約 40 mL H_2/g pretreated DWC （32 mL H_2/g DWC）が得られ，約 5 倍向上した．

以上より，割り箸（他の木質バイオマス：木材チップ，バーク，のこくず，建設廃木材等についても）に適する水素生産プロセスが開発できたと考えられる．現時点では経済的な優位性は確保できていないが，今後さらに詳細にプロセスの最適化を進めることで其れを克服できる可能性は十分あると考えられる．

ACKNOWLEDGEMENT

I would like to express my deepest and sincere gratitude to my advisor, Professor Dr. Tsuyoshi Imai for his kindness and to provide an opportunity to be his advisee. I am also grateful for his valuable supervision, valuable suggestions, supports, encouragement, guidance and criticism throughout the course of my study.

Sincere thanks and appreciation are also addressed to my graduate committee, Professor Dr. Masahiko Sekine, Professor Dr. Masakazu Niinae, Associate Professor Dr. Takaya Higuchi and Associate Professor Dr. Eiichi Toorisaka for their helpful suggestions and criticisms.

I would like to express my sincere gratitude to Associate Professor Dr. Alissara Reungsang, Associate Professor Dr. Koichi Yamamoto, Assistant Professor Dr. Ariyo Kanno and Assistant Professor Dr. Prapaipid Chairattanamanokorn for their excellent comments and suggestions to my work.

We gratefully acknowledge the kind guidance provided by Professor Dr. Rinji Akada and Associate Professor Dr. Hisashi Hoshida for apparatus and advice on HPLC analysis. I am also grateful thank to Professor Dr. Mitsuru Higa and Mr. Masafumi Shibuya for their helps and apparatus on SEM analysis.

This study could not be accomplished without the financial supports from the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT) (Monbukagakusho Scholarship) and MEXT-ARDA under the Asia Core Program (ACP).

Appreciation is also expressed to members of EISEI lab for their practical helps, providing the wonderful environment and friendship during the time of this study. I would like to thank T. Yamamoto for her advising and special helps of doing the experiment of this works, and also thank X. Sun, L.V. Tuan, J. Wei, V.T. Huy, K. Evi, F. Rina, H. Riyanto, Y.P. Devia, W. Suwannasing, and D.T. Loc for their helps and friendship.

Finally, I would like to express my sincere gratitude and appreciation to my beloved parents, my brother and my grandmother, who always be in my heart forever, for their love, motivation, inspiration and encouragement and support throughout of my life.

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LIST OF ABBREVIATIONS

Adj. R^2:	Adjusted coefficients of determination
AFEX:	Ammonia fiber explosion
ANOVA:	Analysis of variance
ATP:	Adenosine triphosphate
BG:	β-glucosidase
$CaCl_2$:	Calcium chloride
CBH:	Exo-glucanase
CBU:	Cellobiase unit
CCD:	Central composite design
CH_4:	Methane
$CH_3CH_2CH_2COOH$:	Butyric acid
CH_3CH_2OH:	Ethanol
CH_3COOH:	Acetic acid
$C_6H_{12}O_6$:	Glucose
CO_2:	Carbon dioxide
$CoCl_2$:	Cobalt chloride
$CuCl_2$:	Cupper chloride
DNS:	Dinitrosalicylic acid
DOE:	Design of experiment
DWC:	Disposable wooden chopsticks
EG:	Endo-glucanase
FD:	Ferredoxin

$FeSO_4.7H_2O$:	Ferrous sulfate heptahydrate
FID:	Flame ionization detector
FPU:	Filter paper unit
GC:	Gas chromatography
H^+:	Hydrogen ion
H_2:	Hydrogen
HAc:	Acetic acid
HBu:	Butyric acid
HCO_3^-:	Bicarbonate
H_2CO_3:	Carbonic acid
HCl:	Hydrochloric acid
H_2O:	Water
HPLC:	High pressure liquid chromatography
HPR:	Hydrogen production rate
HPr:	Propionic acid
HY:	Hydrogen production yield
KH_2PO_4:	Potassium dihydrogen phosphate
K_2HPO_4:	Dipotassium hydrogen phosphate
LPG:	Liquid petroleum gas
$MgCl_2.6H_2O$:	Magnesium chloride hexahydrate
$MnCl_2$:	Manganese chloride
NaOH:	Sodium hydroxide
$NaHCO_3$:	Sodium bicarbonate

NH_4Cl:	Ammonium chloride
$NiSO_4$:	Nickel chloride
O_2:	Oxygen
OFAT:	One factor at a time
ORP:	Oxidation-reduction potential
P:	Hydrogen production potential
PBD:	Plackett- Burman design
PCR-DGGE:	Polymerase chain reaction-denaturing gradient gel electrophoresis
PEG:	Polyethylene glycol
p-value:	Probability unit
R^2:	Coefficients of determination
RSM:	Response surface methodology
SEM:	Scanning electron microscope
TCD:	Thermal conductivity detector
VFAs:	Volatile fatty acids
VSS:	Volatile suspended solids
$ZnCl_2$:	Zinc chloride

CHAPTER I

INTRODUCTION

1.1 Rationale and background

Hydrogen (H_2) plays an important role as an energy carrier in future alternative energy; this is due to the fact that hydrogen is a clean and efficient energy carrier, with zero emissions when burned (Veziroglu and Sümer, 2008; Balat, 2008). There are many conventional methods for producing hydrogen, such as thermochemical process, catalytic reforming of hydrocarbons and electrolysis of water, but these methods are not economical and are mostly nonrenewable (Behera et al., 2004). Production of hydrogen from renewable biomass has several advantages compared to that of fossil fuels (Nath and Das, 2003). Fermentative H_2 production from biomass and wastes are exploited by microorganisms which are capable of converting organic material to acids and alcohols with simultaneous liberation of hydrogen. Bio-H_2 production has a potential for using as sustainable supply energy with low pollution and high efficiency, and it had got special attention in the last decade (Wang et al., 2008).

Recently, lignocellulosic biomass such as agricultural and forest residue has become an interesting potential resource and serves as a renewable feedstock for fermentative hydrogen (H_2) production, due to its costless. It is expected that the cost to produce hydrogen could be lower if waste materials or biomass are used as energy sources. The waste from disposable wooden chopsticks (DWC) was represented as wood waste and investigated in this study. DWC are used daily in Asian countries such as China, Taiwan, Korean and Japan, with an annual consumption of approximately 25 billion pairs (90,000 tons) in Japan made from aspen and white birch wood (Asada et al., 2011). There is significant potential for producing H_2 from DWC to turn waste into energy. Lignocellulosic material comprises cellulose, hemicellulose and lignin and its structure is complex, and usually cannot be directly fermented by microorganisms (Cheng et al., 2011). Thus, it needs to be converted into fermentable sugar. The three main processes of fermentative H_2 production from lignocellulosic biomass are: pretreatment, hydrolysis, and fermentation (Wi et al., 2011). Pretreatment of lignocellulosic biomass is used to remove lignin and partly hydrolyze hemicellulose; while the hydrolysis is used to hydrolyze cellulose and hemicelluloses to

fermentable reducing sugars (Duff andMurray,1996; Hamelinck et al., 2005). Acid and enzymatic hydrolysis is generally applied to convert pretreated biomass into fermentable sugar. However, enzymatic hydrolysis appears to be a better technology in terms of being neither an inhibitor nor byproduct which might have an effect on the downstream processes (El-Zawawy et al., 2011). However, cellulose and hemicellulose are sealed with lignin, delignification of rigid lignin is needed prior to enzymatic hydrolysis. Ester bonds cross-linking between lignin and xylan can be efficiently disrupted by alkaline pretreatment and cause swelling, leading to an increase in internal surface area (Sun and Cheng, 2002). Thus, the accessibility of enzymes to cellulose can be enhanced after alkaline pretreatment. Thermophilic condition has been reported to be appropriate for fermentative H_2 production due to its thermodynamics (Hallenbeck, 2005). It has also been reported that the thermophiles can utilize various types of carbon sources and depress growth of contaminant bacteria. Furthermore, it produces fewer end-products and has higher H_2 production rate over mesophiles (Draphco et al., 2008 and Pawar and Niel, 2013). In developing H_2 production by dark fermentation from lignocellulosic materials, it must be recognized that H_2 production is affected by many factors in each step. Since there are multiple factors affecting H_2 production process, response surface methodology (RSM) was used to design the experiment and determine the optimal conditions for the desirable responses. This method has many advantages over the conventional method (one factor at a time) in terms of time saving and reduction in the work required, together with the opportunity to examine the interaction amongst variables (Bezerra et al., 2008).

1.2 Dissertation objectives

The aim of this study is to develop fermentative H_2 production process from wood material by thermophilic anaerobic mixed culture enriched from hot spring. In order to accomplish the goal of the study, the purposes of the study was divided into four sub-objectives as follow:

1) To optimize the environmental factors for H_2-producing bacteria enriched from hot spring and characterize their microbial community.
2) To investigate the effect of alkaline pretreatment on lignin removal and sugar release from pretreated DWC for using as a substrate on fermentative H_2 production subsequently.
3) To optimize the condition of enzymatic hydrolysis in order to improve the saccharification of pretreated DWC.

4) To optimize the fermentative medium component in order to enhance H_2 production from enzymatic hydrolysate of pretreated by thermophilic anaerobic mixed culture enriched from hot spring.

1.3 Structure of dissertation

The structure of dissertation was divided into six chapters and listed as follow:

- **Chapter I** states the problem, objectives and structure of the dissertation.
- **Chapter II** reviews the literature of the previous studies and introduces the background knowledge of the dissertation.
- **Chapter III** demonstrates the feasibility of using DWC as a feedstock for fermentative H_2 production by an enriched culture from hot spring. The effect of alkaline pretreatment on lignin removal, sugar release from pretreated DWC and inhibitor release were presented in this chapter. Moreover, the optimum environmental factors for fermentation, pH and temperature, the microbial community of thermophilic anaerobic mixed culture obtained from hot spring were also indicated in this chapter.
- **Chapter IV**, according to the Chapter III, the sugar yield obtained from the enzymatic hydrolysis was still low. So that in order to improve the efficiency of the process, enzymatic hydrolysis condition had been optimized in this chapter.
- **Chapter V**, to enhance the H_2 production from enzymatic hydrolysate by thermophilic anaerobic mixed culture, the optimization of medium composition had been described in this chapter.
- **Chapter VI** summarizes the overall results of this study and suggests the idea for future study.

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CHAPTER II

LITERATURE REVIEWS

2.1 Hydrogen

H_2 gas is considered as promising energy carrier that could play a significant role in the reduction of greenhouse gas emissions (Christopher and Dimitrios, 2012). Only water is an end-product on the combustion of H_2 and its provided high energy yield at 120 kJ/g, which is 2.7-fold greater than that of hydrocarbon fuels. Hence, H_2 is regarded as an ideal and alternative energy replacing of fossil fuel in the future, due to low to nonexistent generation of pollutants, and high energy content.

Hydrogen molecule consists of two hydrogen atoms. H_2 is a colorless and odorless gas at room temperature and is the lightest gas, being about 1/14 as dense as air. H_2 easily reacts with oxygen at a wide range of mixing ratios and forms water (combustion). This makes it possible to use H_2 as an alternative energy carrier. The physical and chemical properties of H_2 are listed in **Table 2.1**.

Table 2.1 - The physical and chemical properties of H_2 (Source: Abe, 2014)

Properties	Values
Molecular weight (g/mol)	2.016
Appearance at room temperature	Colorless and odorless
Density (gas) (g/L) at 0°C, 1atm	0.0899
Relative vapor density (air = 1)	0.07
Density (liquid) (g/L) at -253 °C	70.8
Melting point (°C)	-259.35
Boiling point (°C) at 1 atm	-252.88
Solubility in water (cm^3/g)	0.0214
Energy content: Lower heating value (MJ/kg) (MJ/Nm^3) (kJ/mol) Higher heating value (MJ/kg) (MJ/Nm^3) (kJ/mol)	 120 10.76 241 142 12.71 285

Recently, global energy requirements are heavily dependent on fossil fuels such as oil, coal and natural gas. As the depletion of limited fossil fuels is inevitable, there is an urgency to search for replacement source of energy (Das and Veziroglu, 2001). The extensive use of fossil fuel has also created an environmental issue where emission of carbon dioxide (CO_2) during combustion of fossil fuels has caused a global warming effect. For these reasons, researches are looking at alternative fuels that solve both the mentioned problems. H_2 is one of the promising alternative energy; due to H_2 combustion generates high energy content and no pollutants. In comparison with fossil fuel, H_2 has a higher energy yield (Kotay and Das, 2008). The energy content and CO_2 emission of various types of fuel combustion are shown in **Table 2.2**.

Table 2.2 - Energy content and CO_2 emission of various types of fuel combustion (Source: Biomass energy center, UK)

Fuel type	Energy content (MJ/kg)	CO_2 emission (g CO_2/MJ)
Coal (bituminous/anthracite)	29	102.9
Crude oil	42	72.3
Natural gas	38	63.9
Liquid petroleum gas (LPG)	46	66.7
Gasoline	44	72.8
Diesel	42.8	72.6
Bioethanol	27	71.6
Biodiesel	37	75.3
H_2	120	0

2.2 Biological H_2 production processes

Bio-H_2 production offers two major advantages over physical-chemical process in that (1) it utilizes renewable sources and (2) it produces very low or no net level of CO_2 as most of CO_2 produced during carbon metabolism is fixed back and used for the cell growth and energy metabolism. Bio-H_2 production methods fundamentally rely on the presence of a H_2 metabolizing enzyme. Two main enzymes involving in H_2 metabolism are nitrogenase and hydrogenase. Bio-H_2 production can be classified in to three main types as follows:

1. Direct biophotolysis
2. Photo-fermentation
3. Dark fermentation

2.2.1 Direct biophotolysis

H_2 can be produced by many phototropic organisms, such as purple bacteria, green bacteria, Cyanobacteria and several algae with the present of light energy. Microalgae, such as green algae and cyanobacteria, absorb light energy to sprit water to O_2 and generate electrons. The electrons are then transferred to ferredoxin (FD) using the light energy absorbed by photosystem (Saxena et al., 2008). Then, hydrogenase or nitrogenase enzymes catalyze H_2 formation under certain conditions, those enzymes are contained in microalgae and cyanobacteria (Benemann, 1997). The schematic pathway of direct biophotolysis is shown in **Fig. 2.1**. In this process, highest theoretical yield of H_2 production is 12 mole H_2 from 12 mole of water. The reaction for H_2 production from water by direct biophotolysis is presented as following reaction:

$$12H_2O + \text{light energy} \rightarrow 12H_2 + 6O_2 \qquad (2.1)$$

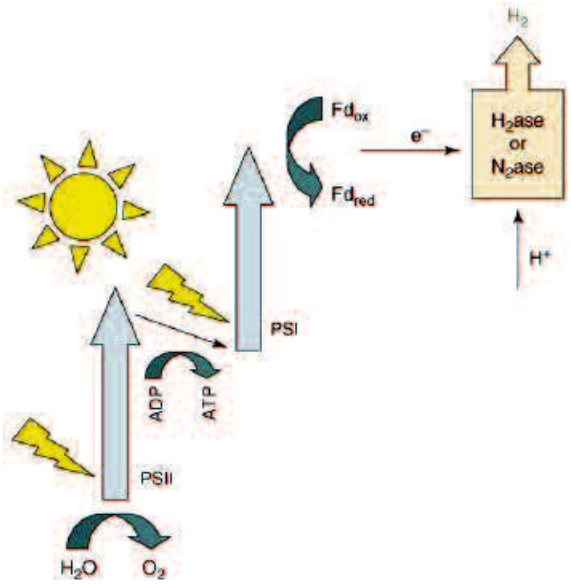

Fig. 2.1 - Schematic pathway of direct biophotolysis (Hallenbeck and Ghosh, 2009)

2.2.2 Photo-fermentation

Non-sulfur purple bacteria utilize organic compounds such as organic acid as a substrate with the present of light energy to generate H_2 under anaerobic condition. These photoheterotrophic bacteria uses captured light energy to produce ATP and high energy electrons (through reverse electron flow) that reduce ferredoxin. ATP and reduced ferredoxin drive proton reduction to H_2 by nitrogenase (Bolton, 1996). The schematic pathway of photo-fermentation is shown in **Fig. 2.2**. In this process, the highest theoretical yield of H_2

production is achieved 4 mol H_2/mol acetic acid. The reaction for H_2 production from glucose by photo-fermentation is presented as following reaction:

$$CH_3COOH + 2H_2O + \text{light energy} \rightarrow 4H_2 + 2CO_2 \quad (2.2)$$

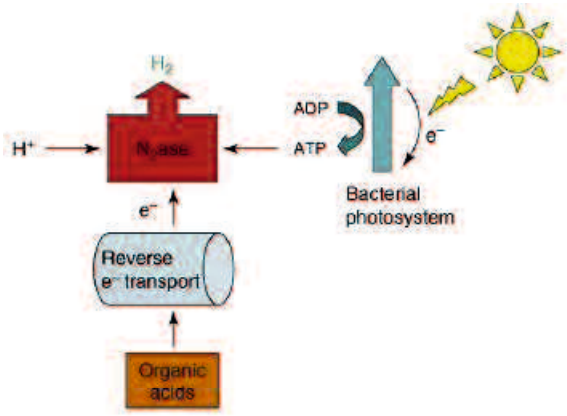

Fig. 2.2 - Schematic pathway of photo-fermentation (Hallenbeck and Ghosh, 2009)

2.2.3 Dark fermentation

A variety of different microbes can be used anaerobically to breakdown carbohydrate-rich substrates to H_2 and other products, principally acids (lactic, acetic, butyric, etc.) and alcohols (ethanol, butanol, etc.). Product distribution can be different dependent upon the microbe, oxidation state of the substrate and environmental conditions (pH, H_2 partial pressure). When acetic acid is the end product, the highest theoretical yield of H_2 production by dark fermentation is obtained at 4 mol H_2/mol glucose. The schematic pathway of dark fermentation is shown in **Fig.2.3**. The reactions for H_2 production from glucose by photo-fermentation is presented as following reaction:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad (2.3)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COOH + 2H_2 + 2CO_2 \quad (2.4)$$

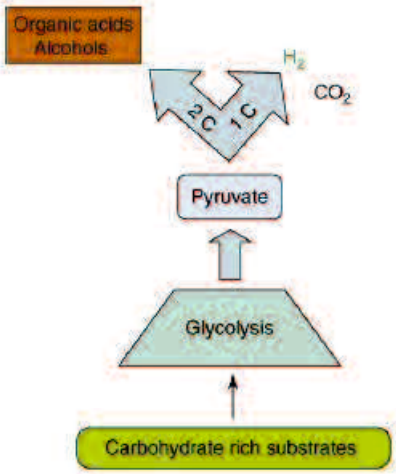

Fig 2.3 - Schematic pathway of dark fermentation (Hallenbeck and Ghosh, 2009)

Table 2.3 - Comparison of different biological processes for H_2 production (Nath and Das, 2004; Hallenbeck and Ghosh, 2009)

Process	Advantages	Disadvantages
Direct biophotolysis	• Can produce H_2 directly from water and sunlight • Simple end-products (H_2 and CO_2)	• Requires high intensity of light • O_2-sensitive hydrogenase • Expensive hydrogen impermeable photobioreactors required
Photo-fermentation	• A wide spectral light energy can be used by these bacteria • Can use different waste materials like distillery effluents, waste etc. • Complete conversion of organic acid wastes to H_2 and CO_2	• Low light conversion efficiencies • High energy demand by nitrogenase • O_2-sensitive nitrogenase
Dark fermentation	• No light energy required • Varity of carbon source like organic waste, energy	• Low H_2 yield obtained • The fermented broth is required to undergo

Process	Advantages	Disadvantages
	crops, lignocellulosic biomass etc. • It produces valuable metabolites such as volatile fatty acids, ethanol as by product • Simple reactor technology required	further treatment before disposal

2.3 Lignocellulosic biomass

Over the last decades, research efforts have focused mainly on bioethanol and biodiesel production. Biofuels have been produced using food crops, such as converting sugar and starch to ethanol, or waste oil to biodiesel. Regarding H_2 fermentation, carbohydrate is a widely used source of carbon, in the form of glucose, xylose, sucrose and starch. The production processes for these biofuels are completely developed technologies, and both commercial and industrial biofuels have been produced from first generation feedstock. Furthermore, they have indirectly caused an increase in food prices and thus contributed to the recent global food crisis. Hence, the production of second generation bio-H_2 by the conversion to bio-H_2 from non-food material or an abundant material such as lignocellulosic biomass is now essential in the move towards renewable energy.

2.3.1 Structure of lignocellulosic biomass

Lignocellulosic materials consist mainly of three polymers: cellulose, hemicellulose and lignin. These polymers are associated with each other in a hetero-matrix to different degrees and varying relative composition depending on the type, species and even source of the biomass (Carere et al. 2008; Chandra et al. 2007). Schematic structure of lignocellulosic biomass is illustrated on **Fig. 2.4**. **Table 2.4**, taken from Garrote et al. (1999), shows a classification for lignocellulosic feedstocks and their average compositions.

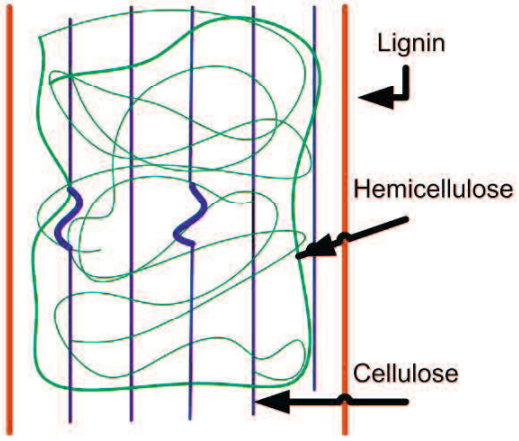

Fig. 2.4 - Schematic structure of lignocellulosic biomass

Table 2.4 - Cellulose, hemicellulose, and lignin contents in various lignocellulosic biomass (Garrote et al., 1999)

Raw material	Cellulose	Hemicellulose	Lignin
Hardwoods			
White birch	41	36.2	18.9
Poplar aspen	50.8-53.3	26.2-28.7	15.5-16.3
Red maple	44.1	29.2	24
Eucalyptus viminalis	41.7	14.1	31
Softwoods			
Pinus banksiana	41.6	25.6	28.6
Pinus pinaster	42.9	17.6	30.2
fir	43.9	26.5	28.4
Agricultural residues			
Corn cobs	33.7-41.2	31.9-36	6.1-15.9
Sugar cane bagasse	40-41.3	27-37.5	10-20
Wheat straw	32.9-50	24-35.5	8.9-17.3
Rice straw	36.2-47	19-24.5	9.9-24
Corn stalks	35-39.6	16.8-35	7-18.4
Barley straw	33.8-37.5	21.9-24.7	13.8-15.5
Cotton stalks	38.4-42.6	20.9-34.4	21.5

2.3.1.1 Cellulose

Cellulose $(C_6H_{10}O_5)_x$, the main component of lignocellulosic biomass, is a polysaccharide that was formed by a linear chain of D-glucose linked each other unit with β-(1,4)-glycosidic bonds. The cellulose chains are formed together to make cellulose fibrils. Cellulose fibers are linked by a number of intra-and intermolecular hydrogen bonds (Li et al., 2010). Therefore, cellulose is insoluble in water and most organic solvents (Swatloski et al., 2010).

2.3.1.2 Hemicelluloses

Hemicelluloses $(C_5H_8O_4)_m$, located in between lignin and cellulose, are heterogeneous branched biopolymers containing pentoses (β-D-xylose, α-L-arabinose), hexoses(β-D-mannose, β-D-glucose, α-D galactose) and/or urgonic acids (α-D-glucuronic, α-D-4-O-methylgalacturonic and α-D-galacturonic acids) (Girio et al., 2010). Their structure is branch with short lateral chain, amorphous, and has low molecular weight, so that hemicelluloses are easily hydrolyzed under severe condition (Li et al., 2010). In order to increase the digestibility of cellulose, large amounts of hemicelluloses must be removed as they cover cellulose fibrils limiting their availability for the enzymatic hydrolysis (Agbor et al., 2011). Hemicelluloses are relatively sensitive to operation condition; therefore, parameters such as temperature and retention time must be carefully optimized to avoid the formation of hemicellulose degradation products such as furfurals and hydroxymethyl furfurals which have been reported to inhibit the fermentation process (Palmqvist and Hahn-Hägerdal 2000a; 2000b). For this reason, to maximize sugar recovery, pretreatment are needed to accommodate severity of its condition and depending upon type of pretreatment method is used hemicellulose could be obtained either as a solid fraction or a combination of both solid and liquid fractions (Chandra et al. 2007).

2.3.1.3 Lignin

Lignin $[C_9H_{10}O_3(OCH_3)0.9–1.7]_n$ is an aromatic polymer synthesized from phenylpropanoid precursors. The major chemical phenylpropane units of lignin consisting primarily of syringyl, guaiacyl and *p*-hydroxy phenol are linked together to make a complicated matrix (Demirbas, 2008). Lignin is present in plant cell walls and confers a rigid, impermeable, resistance to microbial attack and oxidative stress (Hendricks and Zeeman 2009). Lignin is generally accepted as the ‘glue’ that binds the different components of lignocellulosic biomass together, thus making it insoluble in water. Because of its close

association with cellulose microfibrils, lignin has been identified as a major deterrent to enzymatic and microbial hydrolysis of lignocellulosic biomass (Avgerinos and Wang 1983). Different feedstocks contain different amount of lignin that must be removed via pretreatment to enhance biomass digestibility. Delignification (extraction of lignin by chemicals) causes biomass swelling, disruption of lignin structure, increases in internal surface area, and increased accessibility of cellulolytic enzymes to cellulose fibers. (Brownell and Saddler 1987; Converse 1993; Lynd et al. 2002). Lignin removal is required prior further hydrolysis and/or fermentation processes.

2.3.2 Wood material

In some regions, there is an abundance of woody residues and solid wood waste (forest biomass) generated from forest management activities and forest products manufacturing. Wood residues include chip, bark, and sawdust that produced within various wood-processing industries including sawmills, furniture factories, and other industries. Moreover, wood waste in municipal solid waste such as disposable wooden chopsticks was generated the most in East Asian countries such as Taiwan, China. The annual average amount of waste produced by the DWC was approximately 90,000 tons in Japan (Asada et al., 2011). It is possible to generate valuable products from wooden biomass that in many cases is disposed at landfills or is incinerated (Shabani et al., 2013). Since a large amount of wooden waste is generated annually, there is the possibility that wood waste can be used as a renewable low-cost feed stock for fermentative H_2 production in terms of conversion of waste to energy.

2.4 Pretreatment of lignocellulosic biomass

The complex structure of lignocellulosic biomass makes it difficult to utilize as a feedstock for bio-H_2 production. It requires extensive pretreatment of the cellulosic feedstock in order to convert cellulose and hemicelluloses to fermentable sugars. The pretreatment generally includes pretreatment and hydrolysis: pretreatment of lignocellulosic biomass is used to remove lignin and partly hydrolyze hemicellulose; while the hydrolysis is used to hydrolyze cellulose and hemicelluloses to fermentable reducing sugars (Duff and Murray, 1996; Hamelinck et al., 2005). **Fig. 2.5** shows overall production processes of bio-H_2 from lignocellulosic biomass.

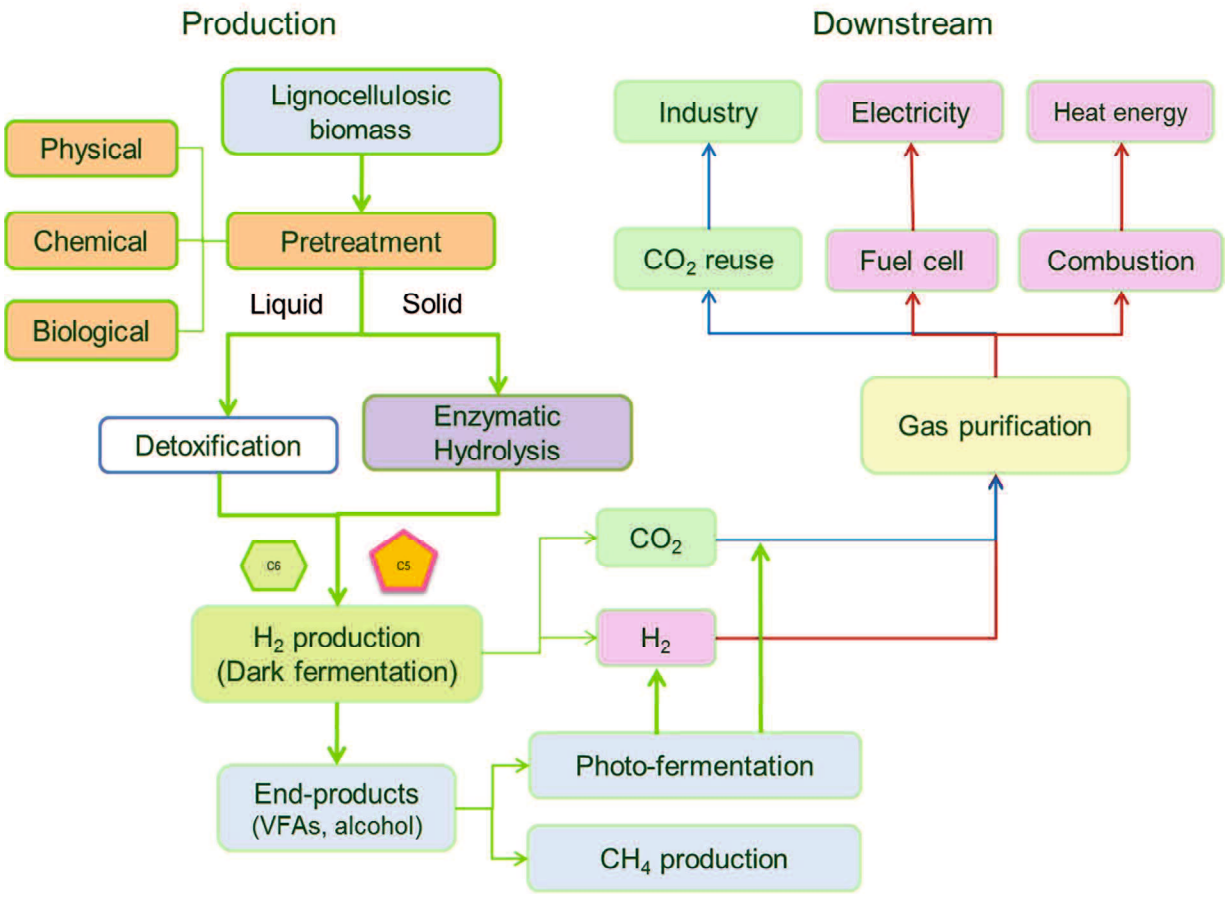

Fig. 2.5 - Overall production processes of bio-H_2 from lignocellulosic biomass

In order to achieve the efficiency of pretreatment step in bio-H_2 production, the pretreatment process of lignocellulosic biomass should meet following requirement (Galbe and Zacchi, 2007);

- Result in high yields of fermentable reducing sugars.
- Result in high digestibility of the cellulose in the subsequent enzymatic hydrolysis.
- Produce no or very limited amounts of sugar and lignin degradation products which later inhibit the subsequent fermentation (Sun and Cheng, 2002). The pretreatment liquid should be possible to ferment without detoxification.
- Result in high solids concentration as well as high concentration of liberated sugars in the liquid fraction.
- Require a low energy demand or be performed in a way so that the energy can be re-used in other process steps as secondary heat.
- Require low capital and operational cost.

Several methods have been introduced for pretreatment of lignocellulosic materials prior to enzymatic hydrolysis. These methods are classified into 1) Physical pretreatment, 2) Physico-chemical pretreatment, 3) Chemical pretreatment and 4) Biological pretreatment. **Table 2.5** summarizes the effect of difference pretreatment methods on the chemical composition and structure of lignocellulosic biomass and their limitation.

2.4.1 Alkaline pretreatment

Some bases can be used for the pretreatment of lignocellulosic materials, and the effect of alkaline pretreatment depends on the lignin content of the biomass (Fan et al., 1987). Alkaline pretreatment processes utilize lower temperatures and pressures than other pretreatment technologies (Mosier et al., 2005). Alkali pretreatment can be carried out at ambient conditions, but pretreatment times are on the order of hours or days rather than minutes or seconds. Nevertheless, possible loss of fermentable sugars and production of inhibitory compounds must be taken into consideration to optimize the pretreatment conditions.

Dilute NaOH treatment of lignocellulosic materials has been found to cause swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure (Taherzadeh and Karimi, 2008). Dilute NaOH pretreatment was found to be effective for the hydrolysis of straws with relatively low lignin contents of 10-18% (Bjerre et al., 1996). Wan et al. (2011) pretreated soy bean straw with NaOH (4–40 g/100g dry straw) at ambient condition and achieved glucose yield of 64.55% and xylan removal of upto 46.37%. In another study, costal Bermuda grass was pretreated with 0.75% NaOH solution during 15 min, and a total reducing sugar yield of 71% was achieved. In addition, the overall conversion efficiencies for glucan and xylan were 90.43% and 65.11%, respectively (Wang et al., 2010). The digestibility of NaOH-treated hardwood was reported to increase from 14% to 55% with a decrease of lignin content from 24-55% to 20%.

Table 2.5 - Effect of difference pretreatment methods on the chemical composition and structure of lignocellulosic biomass and their limitation (Mood et al., 2013)

Pretreatment method	Increase surface area	Cellulose de-crystallization	Hemicellulose removal and solubilization	Lignin removal	Inhibitor formation	Limitation
Physical pretreatment						
Milling, extrusion and microwave	++	++	-	-	-	High energy consumption
Physico-chemical pretreatment						
Liquid hot water	++	ND	++	+/-	+	High temperature, need to add alkaline to control pH
Steam explosion	++	-	++	+/-	++	Incomplete disruption of lignin-carbohydrate matrix, formation of toxic component
CO_2 explosion	++	-	++	-	-	High pressure requirement, does not effect on lignin and hemicellulose
Ammonia fiber explosion (AFEX)	++	++	+	++	+/-	High pressure requirement, low efficiency for high lignin content biomass, high cost of ammonia
Wet oxidation	++	+	++	++	+/-	High cost of oxygen and catalyst
Chemical pretreatment						
Acid	++	-	++	+	++	Equipment corrosion, degrading produce sugar, neutralization of pretreated slurry
Alkaline	++	-	+	++	+/-	Long pretreatment residence time, neutralization of pretreated slurry
Ionic liquid	++	++	+	+	-	High cost of ionic liquid
Organosolv	++	ND	++	++	-	Recovery and recycle of solvent by evaporation, high cost
Ozonolysis	++	ND	-	++	-	Large amount of ozone requirement, expensive process
Biological pretreatment						
Fungi and actinomycetes	++	+	+/-	++	-	Low hydrolysis rate, large space requirement, need to control condition of microorganism growth

2.5 Enzymatic hydrolysis

Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific (Beguin and Aubert, 1994). The products of the hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is low compared to acid hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45-50°C) and does not have a corrosion problem (Duff and Murray, 1996). Cellulases are usually a mixture of several enzymes. At least three major groups of cellulases are involved in the hydrolysis process (**Fig. 2.6**): (1) endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC 3.2.1.4.) which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends; (2) exoglucanase or cellobiohydrolase (CBH, 1,4-b-D-glucan cellobiohydrolase, or EC 3.2.1.91.) which degrades the molecule further by removing cellobiose units from the free chain-ends; (3) β-glucosidase (BG, EC 3.2.1.21) which hydrolyzes cellobiose to produce glucose (Wilson, 2011). During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented by bacteria to bio-H_2.

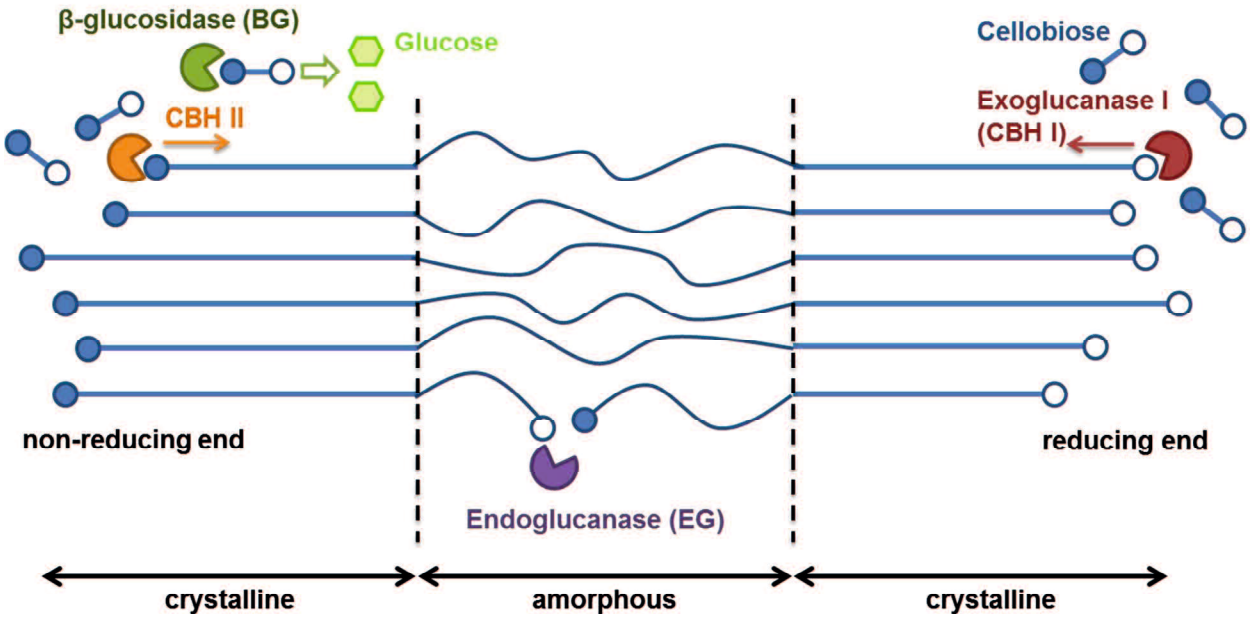

Fig. 2.6 - Enzymatic hydrolysis of cellulose.

The factors that affect the enzymatic hydrolysis of cellulose include substrates loading, cellulase activity, and reaction conditions.

2.5.1 Substrates loading

Substrate concentration is one of the main factors that affects the yield and initial rate of enzymatic hydrolysis of cellulose. At low substrate levels, an increase of substrate concentration normally results in an increase of the yield and reaction rate of the hydrolysis (Cheung and Anderson, 1997). However, high substrate concentration can cause substrate inhibition, which substantially lowers the rate of the hydrolysis, and the extent of substrate inhibition depends on the ratio of total substrate to total enzyme (Huang and Penner, 1991). Penner and Liaw (1994) reported that the optimum substrate to enzyme ratio was 1.25 g of the microcrystalline substrate Avicel pH 105 per FPU of the cellulase from *T. reesei*. The susceptibility of cellulosic substrates to cellulases depends on the structural features of the substrate including cellulose crystallinity, degree of cellulose polymerization, surface area, and content of lignin. Lignin interferes with hydrolysis by blocking access of cellulases to cellulose and by irreversibly binding hydrolytic enzymes. For example, Boussaid and Saddler (1999) measured the minimum enzyme required to degrade a substrate to completion. With Avicel, they were able to have enzyme loadings of 40 mg/g cellulose (or 40 FPU/g cellulose), while 60 FPU/g was required for delignified kraft pulp. However, even enzyme loadings of 750 FPU/g cellulose were not able to fully hydrolyse the pulp which contained 28% lignin. However, removal of lignin can dramatically increase the hydrolysis rate (McMillan, 1994).

2.5.2 Cellulase dosage

Enzymatic hydrolysis of cellulose consists of three steps: adsorption of cellulase enzymes onto the surface of the cellulose, the biodegradation of cellulose to fermentable sugars, and desorption of cellulase. Increased enzyme loadings may lead to increased hydrolysis, but only up to a certain point, after that hydrolysis slows down due to various factors. The efficiency ratio will give an indication of the limit of protein loading required for optimal degradation and economy. At high enzyme loadings, the relative number of binding sites is reduced and enzymes may start competing for the same binding sites, leading to a reduction in the overall rate (Banerjee et al., 2010). According to Sun and Cheng (2002), cellulase loadings of 7–33 FPU/g substrate is generally used, depending on the specific substrate.

2.5.3 End-product inhibitors

It has a report that enzymes are inhibited by the products of their reaction. Cellulases are inhibited by cellobiose (Gruno et al., 2003), while β-glucosidase is inhibited by glucose

(Andric et al., 2010; Holtzapple et al., 1990). For this reason, excess β-glucosidase is generally added to cellulases in bioconversion processes to prevent inhibition of cellulases. Use of a cellulase mixture from different microorganisms or a mixture of cellulases and other enzymes in the hydrolysis of cellulosic materials has been extensively studied (Excoffier et al., 1991; Xin et al., 1993). The addition of β-glucosidases into the *T. reesei* cellulases system achieved better saccharification than the system without β-glucosidases (Excoffier et al., 1991; Xin et al., 1993). β-glucosidases hydrolyze the cellobiose which is an inhibitor of cellulase activity. A nearly complete saccharification of steam-explosion pretreated *Eucalyptus viminalis* chips (substrate concentration of 6% and enzyme loading of 10 FPU/g cellulose) was obtained using a cellulase mixture of commercial Celluclast and Novozyme preparations (Ramos et al., 1993).

2.5.4 Surfactant

Cellulase activity decreases during the hydrolysis. The irreversible adsorption of cellulase on cellulose is partially responsible for this deactivation (Converse et al., 1988). Addition of surfactants is one of the most common ways to prevent nonproductive adsorption of enzymes to their substrate and thereby improve hydrolysis. Sun and Cheng (2002) gives an overview of the literature in this regard and concluded that non-ionic surfactants were the most suitable for improving cellulose hydrolysis, a conclusion that was supported by Eriksson et al. (2002). Eriksson et al. (2002) proposed that non-ionic surfactants, such as Tween and Triton, may improve hydrolysis by hydrophobic interaction with lignin, which caused a release of the non-productively adsorbed enzymes (**Fig. 2.7**). This was confirmed in a study of cellulase binding on lignin by Tu et al. (2009), who concluded that the Tween 80 displayed competitive binding with the cellulases, thus reducing cellulase adsorption to the lignin.

To improve the yield and rate of the enzymatic hydrolysis, research has focused on optimizing the enzymatic hydrolysis process.

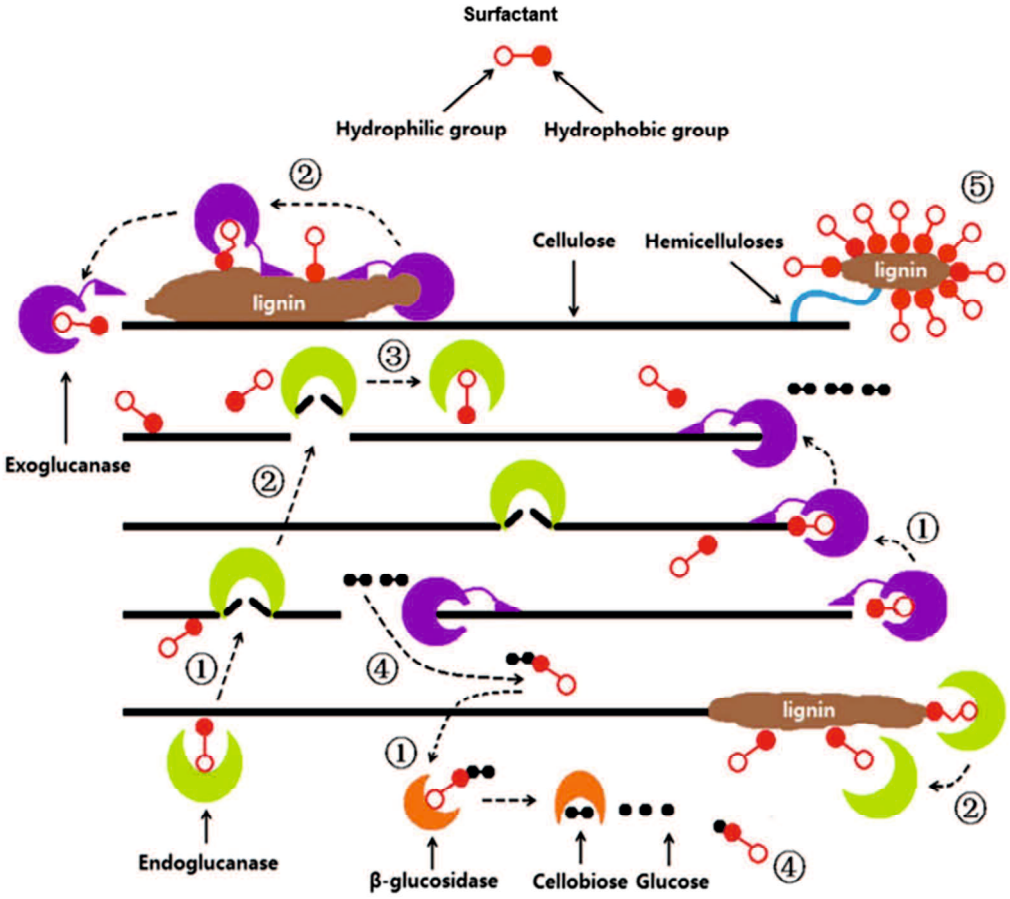

Fig. 2.7 – The role of surfactant in enzymatic hydrolysis of lignocellulosic biomass (1) Enzymes and substrates are attracted together by the surfactant; (2) Surfactant promotes the release of enzymes non-productively binding on the substrate; (3) Surfactant led to a higher recovery of enzymatic activity at the end of hydrolysis; (4) Surfactant homogenize organic matter in solution with its hydrophilic and hydrophobic groups; and (5) the hydrophobic interaction between surfactant and lignin (modified from Feng et al., 2013).

2.6 Fermentative H_2 production

2.6.1 Thermophilic H_2 producing-bacteria

Thermophilic H_2 producing-bacteria are mostly obligate anaerobes found in various geothermal regions of the earth, such as hot springs. Thermophiles growing at elevated temperatures (generally from 40 - 70 °C) were especially attractive recently. There are many advantages of thermophilic bacteria over mesophile bacteria as listed: [1] the higher temperature is thermodynamically favorable for biofuel production (Raj et al., 2012). [2] Thermophilic bacterial growth at elevated temperatures decreases the possibility of contamination by unwanted microorganisms such as methanogens and solvent-producing

bacteria (Blumer-Schuette et al., 2008). [3] Most of the thermophilic bacteria have the capability of fermenting a board range of substrates to produce H_2 (Chang and Yao, 2011). [4] Thermophilic fermentation compared with mesophiles produces less variety of end products (vanNiel et al., 2003) and higher H_2 yields (Kadar et al., 2004).

2.6.2 Factor affecting on H_2 production

2.6.2.1 Temperature

H_2 producing-bacteria are mostly categorized in two main groups: Mesophilic and thermophilic bacteria whose favorable ranges of temperature are 30 - 40 and 45 - 55°C, respectively. It is reported that increase of temperature generally improves H_2 production rate. However, there is no exact optimum temperature in bio-H_2 production. The optimum temperature for producing H_2 via dark fermentation depends on the type of H_2 producing-bacteria and also the carbon source used. For example, Wu et al. (2005) reported the optimum temperature in the range of 37 - 45°C for the pure cultures Clostridium or Enterobacter species; whereas, the optimum temperature for *Thermoanaerobicterium thermosaccharolyticum* PSU-2 was found at 60°C by Sompong et al. (2008). Lin and coworkers reported a 260% increase in maximum H2 production rate (HPR) by means of temperature variation from 45 - 55°C, while they achieved only 100% improvement of maximum H_2 production rate by increasing temperature from 30 - 40°C (Lin et al., 2008)

2.6.2.2 pH

pH is one of the most important factors in dark fermentation that affects to hydrogenase activity and metabolic pathways (Tang et al., 2008). The optimal pH in terms of bio-H_2 production is within a range of 5.0 - 7.0 which probably favors the activity of the hydrogenases and is also suitable for microbial development in dark fermentation (Li and Fang, 2007). Optimal initial pH for H_2 production from hydrolyzates has been between 6.5 and 7 with enrichment cultures from cow dung compost (Zhang et al., 2007; Cui et al., 2009), 5.5 with *Clostridium butyricum* (Pattra et al., 2008), and 8.0 with dairy manure bacteria (Pan, et al., 2010). However pH other than the optimum has been shown to suppress the H_2 yields (Kumar and Das, 2000a). Thus, it is important to control the pH in order to produce higher H_2 production.

2.6.2.3 Reducing agent

In the anaerobic process, a reducing agent like L-cysteine is required to reduce the oxidation-reduction potential (ORP), it's commonly used in anaerobic culture media. In addition, L-cysteine could increase cell growth rate (Song and Logan, 2004). This was proved by previous studies only by batch mode. Yuan et al. (2008) and Bao et al. (2013) investigated the addition of L-cysteine and found that it could enhance H_2 productivity, bacteria growth and substrate utilization.

2.6.2.4 Buffer

During the dark fermentation, volatile fatty acids (VFAs) were also produced to accompany H_2; the pH in the culture medium was dropped and caused an adverse effect on cell growth and H_2 production. A buffer needs to be added to the culture medium in order to avoid the rapid decrease of pH in the process. Thus, most of the dark fermentation cultures used bicarbonate buffer to avoid sharp decrease in pH (Chang et al., 2002; Khamtib et al., 2011; Boonsayompoo and Reungsang, 2013). However, bicarbonate buffer leads to additional CO_2 formation due to interaction of HCO_3 and acidic metabolites (Eq. (2.5)), resulting in a significant increase in CO_2/H_2 ratio:

$$HCO_3^- + H^+ \rightarrow H_2CO_3 \rightarrow H_2O + CO_2 \quad (2.5)$$

To decrease the CO_2/H_2 ratio for less CO_2 emission and easier downstream purification of H_2, this study used phosphate buffer to replace bicarbonate buffer that has been applied in the majority of dark H_2 fermentation experiments (Oh et al, 2003). A phosphate buffer not only has the buffer capacity but is also used as a phosphate source for microbial growth (Lin and Lay, 2004).

2.6.2.5 Nitrogen source

The nitrogen source is an essential component for proteins, nucleic acids, and enzyme synthesis (Wang and Wan, 2009), which is necessary for bacterial activity and growth. Nitrogen at an optimal concentration is beneficial to H_2 production, while at higher concentrations can inhibit the process performance by affecting the intracellular pH of the bacteria or inhibiting specific enzymes related to H_2 production (Bisaillon et al., 2006 ; Chen et al., 2008). At elevated nitrogen levels, the metabolic path might lead towards ammonification, where the protons get consumed instead of forming H_2 (Salerno et al., 2006).

2.6.2.6 Metal ions

Trace metals are required for the activation or function of many enzymes and coenzymes related to energy metabolism and are also essential for the cell growth of many microorganisms. Each of the trace metals has a specific biochemical function in the cell during metabolism, and a change in their concentration may alter that metabolic function. Srikanth and Venkata Mohan (2012) reported that magnesium plays an important role in the metabolic processes for substrate utilization. Magnesium is also a component of cell walls and cell membranes and a cofactor of many enzymes involved in the glycolysis pathway (Wang et al., 2007). Iron and Nickel are the main components on the active site of hydrogenase, which is the key enzyme in dark fermentation (Vignais and Billoud, 2007 and Hallenbeck and Ghosh, 2009). Trace elements are required for the activation or function of several enzymes and coenzymes related to energy metabolism e.g., manganese (Srikanth and Venkata Mohan, 2012), zinc, copper (Zheng and Yu, 2004), and calcium (Yuan et al., 2010).

2.7 Design of experiment (DOE)

Optimization has been carried out in attempts to improve bio-H_2 production. Rates and yields of H_2 production, as for many other bioprocesses, are a function of several variables, including pH, temperature, substrate concentration and nutrient availability, among others. Conventionally, process optimization has been carried out by monitoring the influence of one factor at a time on an experimental response. While only one parameter is changed, others are kept at a constant level. This optimization technique is called one-factor-at-a-time (OFAT). Its major disadvantage is that it does not include the interactive effects among the variables studied. As a consequence, this technique does not depict the complete effects of the parameter on the response (Montgomery, 2013). Another disadvantage of the one-factor optimization is the increase in the number of experiments necessary to conduct the research, which leads to an increase of time and expenses as well as an increase in the consumption of reagents and materials (Bezerra et al., 2008). On the contrary, the statistical methods are believed to be more effective and powerful in screening key factors from a multivariable system and optimizing fermentation conditions (Tanyildizi et al., 2005; Liu and Wang, 2007), and they are also more time-saving and error-proof in determining the effect of parameters (Abdel-Fattah and Olama, 2002). Some statistical methods including the Plackett- Burman design and response surface methodology (RSM) with various designs have been successfully employed for optimization in some bioprocesses, such as enzymatic hydrolysis

(Singh and Bishnoi, 2012), critical medium components (Liu and Wang, 2007), bio-H_2 production (Pan et al., 2008; Cao et al., 2010; Boonsayompoo and Reungsang; 2013).

Montgomery (2013) suggested that there are three main steps for process optimization. First of all, Factorial design such as fractional factorial design or Plackett–Burman design is used to screen the key factors of a fermentative H_2 production process for further study. And then, the method of steepest ascent is used to approach the vicinity of the optimal conditions. Subsequently, central composite design or Box–Behnken design for response surface methodology can be used to estimate the relationship between a response and these key factors at the vicinity of optimum and then locate the optimal conditions based on a second-order polynomial model.

2.7.1 Plackett-Burman design (PBD)

Plackett–Burman design, which is a two-level fractional factorial design developed by Plackett and Burman, has been extensively used to screen important factors for further investigation (Kennedy and Krouse, 1999). In addition, the number of runs for a PBD is equal to a multiple of 4. PBD can examine up to k = N - 1 factors in an experiments with N runs and it works for all such N up to 100, except for 92 (Kuehl, 2000). A first-order polynomial model (Eq. (2.6)) is usually used to describe the effects of various factors on it based on the experimental results from a PBD.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i \qquad (2.6)$$

where y is the response, b_0 is the constant and b_i is the linear coefficient, and x_i is the coded factor levels.

Based on the analysis of variance (ANOVA) of the estimated model, the significant factors can be identified (Plackett and Burman; 1946).

2.7.2 Path of steepest ascent

Frequently, the initial estimate of the optimal conditions for a bioprocess is far from the actual optimum. Thus, the second step for optimization is to locate the region of factor levels that produce optimal conditions. The factors screened by the Plackett–Burman design can be further investigated using this method. In order to obtain the path of steepest ascent for various factors, a first-order polynomial model (Eq. (2.6)) is usually used to fit the experimental data obtained from a factorial design such as a Plackett–Burman design. The

path of steepest ascent is perpendicular to the contour plots of the response based on the estimated first-order polynomial model, and moves b_i units in the x_i direction for every b_j units in the x_j direction. Equivalently, the path has a movement of b_j/b_i units in x_j for every 1 unit movement in x_i. **Fig. 2.8** shows the contour plot of a response with varying only two factor levels, while keeping other factor levels constant, and the corresponding path of steepest ascent. The path of steepest ascent starts from the design center of the factorial design building the first-order polynomial model and ends until no further improvement can be achieved in the response, which indicates that the region of optimal response is in the neighborhood of that condition (Montgomery, 2013).

2.7.3 Central composite design (CCD)

Once the region of optimal response is identified by the method of steepest ascent, it is often necessary to characterize the response in that region. Central composite design is widely used experimental designs for response surface methodology to estimate a second-order polynomial approximation to a response in that region.

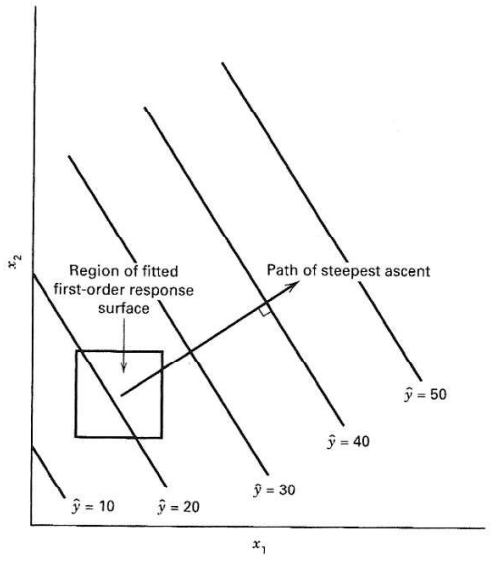

Fig. 2.8 - First-order response surface and path of steepest ascent (Montgomery, 2013).

The central composite design was presented by Box and Wilson (1951). This design consists of the following parts: (1) a full factorial or fractional factorial design; (2) an additional design, often a star design in which experimental points are at a distance α from its center; and (3) a central point. **Fig. 2.9** illustrates the full central composite design for

optimization of two and three variables. Full uniformly routable central composite designs present the following characteristics:

(1) Require an experiment number according to N= k^2 +2k+cp, where k is the factor number and (cp) is the replicate number of the central point;

(2) α-values depend on the number of variables and can be calculated by α=2(k-p)/4. For two, three, and four variables, they are, respectively, 1.41, 1.68, and 2.00;

(3) All factors are studied in five levels (-α, -1, 0, +1, +α).

For response surface methodology, a second-order polynomial model (Eq. (2.7)) is usually proposed to describe the effects of various factors on a response based on experimental results from a central composite design or design.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j \qquad (2.7)$$

where y is the response, β_0 is the constant and β_i is the linear coefficient, β_{ii} is quadratic coefficient, β_{ij} is the interactive coefficient and x_i is the coded factor level.

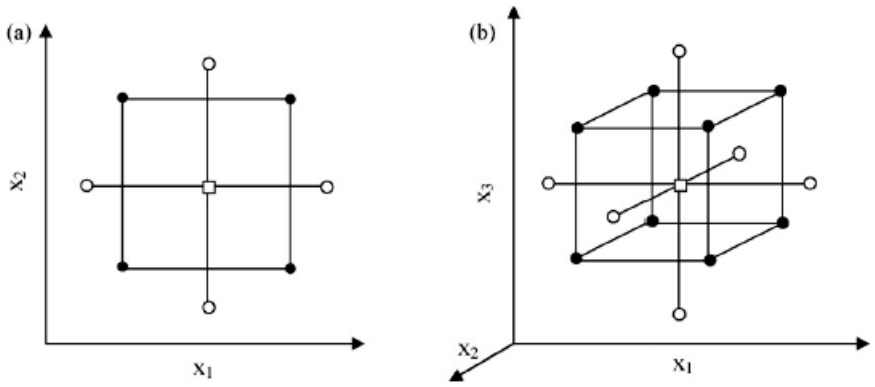

Fig. 2.9 - Central composite designs for the optimization of: (a) two variables (α= 1.41) and (b) three variables (α= 1.68). (●) Points of factorial design, (○) axial points and (□) central point

Based on the ANOVA of the estimated model, terms which have significant effects on the response can be determined. In addition, with the aid of the regression model, the optimal response can be estimated by calculating the derivatives of the model.

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CHAPTER III

DELIGNIFICATION OF DISPOSABLE WOODEN CHOPSTICKS WASTE FOR FERMENTATIVE HYDROGEN PRODUCTION BY AN ENRICHED CULTURE FROM A HOT SPRING

3.1 Introduction

H_2 is considered to be a promising alternative fuel for the future. Lower CO_2 emissions result from improved combustion, leading to the replacement of hydrocarbon fuel consumption. Lignocellulosic materials, including agricultural residues, forestry, and municipal wastes, are the Earth's most abundant and available renewable resources (Saratale et al., 2008). Disposable wooden chopsticks (DWC) waste is becoming interesting as a feedstock for H_2 production, because the original chopsticks are made from aspen or white birch wood and are widely used in restaurants, school cafeterias, supermarkets and convenience stores all over Japan. Approximately 90,000 tons of DWC waste are disposed of each year (Asada et al., 2011) and could benefit from both energy recovery and waste utilization by converting waste to energy. To date, there have been limited reports on the possibility of using DWC waste as a feedstock for fermentative H_2 production. Lignocellulosic material comprises cellulose, hemicellulose and lignin and its structure is complex, and usually cannot be directly fermented by microorganisms (Cheng et al., 2011). Because cellulose and hemicellulose are sealed with lignin, delignification of rigid lignin is needed prior to enzymatic hydrolysis. Ester bonds cross-linking between lignin and xylan can be efficiently disrupted by alkaline pretreatment and cause swelling, leading to an increase in internal surface area (Sun and Cheng, 2002). Thus, the accessibility of enzymes to cellulose can be enhanced after alkaline pretreatment. Dilute alkaline pretreatment has been reported effective on agricultural residues such as, cotton stalk, rice straw and rapeseed straw (Silverstein et al., 2007, Narra et al., 2012 and Choi et al., 2013). Considering reduction of the cost of operation and other environmental aspects, decreasing concentration of chemical usage is one possible way. However, there is limited information of dilute alkaline pretreatment on wood materials. In addition, Knill and Kennedy (2003) and Tanksale et al. (2010) revealed that cellulose and hemicellulose could resist alkaline pretreatment but it starts degrading at temperatures higher than 170°C and 100°C, respectively. Since hemicellulose could be converted to pentose sugars e.g., xylose and arabinose which are possibly used as

substrate for fermentative H_2 production. In order to prevent hemicellulose degradation during delignification step, it would be better to avoid performing pretreatment process at temperature greater than 100°C. Moreover, the advantages of low temperature alkaline pretreatment are that it does not require specialized equipment with heat/pressure resistant and it consumes less energy.

Recently, thermophilic condition has been reported to be appropriate for fermentative H_2 production due to its thermodynamics (Hallenbeck, 2005). It has also been reported that the thermophiles can utilize various types of carbon sources and depress growth of contaminant bacteria. Furthermore, it produces fewer end-products and has higher H_2 production rate over mesophiles (Draphco et al., 2008 and Pawar and Niel, 2013). There are many hot springs in Japan, so thermophilic H_2-producing bacteria are expected to be obtained from hot spring in this study. In developing H_2 production by dark fermentation from lignocellulosic materials, it must be recognized that fermentative H_2 production is a very complex process, affected by many factors such as initial pH and temperature because these factors influence both the activities of H_2-producing bacteria and the fermentative production mechanism (Tang, et al., 2008). Therefore, the optimization of culture conditions must be conducted to facilitate fermentative H_2 production.

The aim of this study was to demonstrate the feasibility of using DWC as a feedstock for fermentative H_2 production by an enriched culture from hot spring. A dilute alkaline pretreatment step was developed to remove lignin prior to enzymatic hydrolysis. The effects of NaOH concentration, temperature and retention time on delignification and carbohydrate release were investigated and the environmental factors of the culture conditions, initial pH and temperature, were optimized. Furthermore, the enzymatic hydrolysate from pretreated DWC was demonstrated to be a suitable substrate for H_2 production. In addition, the bacterial community presented in the enriched hot spring culture was analyzed by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE).

3.2 Materials and methods

3.2.1 Alkaline pretreatment of DWC

DWC waste was collected from the cafeteria at the Faculty of Science and Engineering, Yamaguchi University, Yamaguchi Prefecture, Japan. DWC were soaked in tap water overnight and washed with detergent, then dried at 45°C overnight. Dry DWC were ground in a mill (LM-Plus, Osaka Chemical, Japan) and passed through a 2 mm screen, then

stored at room temperature in sealed container. The effects of pretreatment factors (NaOH concentration, temperature and retention time) were investigated by 3×2×3 full factorial design. Tested conditions were NaOH concentrations of 0%, 1%, and 2% (w/v), temperatures of 50°C and 100°C, and retention times of 30, 45, and 60 min. Ten percent of ground DWC (dry basis w/v) was dispersed in 20 mL of a desired alkaline solution and incubated at desired temperatures for given retention times. Pretreated DWC (cellulose fraction) were filtered in a Buchner funnel fitted with Whatman No. 1 paper, and washed with tap water until neutral, dried at 45°C and analyzed for the remaining acid-insoluble lignin. The liquid fraction was collected to measure total phenolic compounds released by alkaline pretreatment.

3.2.2 Enzymatic hydrolysis

Pretreated DWC (7% dry basis w/v) was hydrolyzed by 20 $U/g_{pretreated\ DWC}$ Cellulase (Celluclast 1.5L, Sigma, USA) in 10 mL of 0.1 mol/L sodium citrate buffer pH 4.8, incubated at 50°C for 12 hr. The hydrolysis process was terminated by boiling for 10 min. After cooling down to room temperature, liquid and solid fractions were separated by centrifuge at 4,620×g for 20 min. Supernatant was collected as the enzymatic hydrolysate and the released carbohydrates analyzed by high pressure liquid chromatography (HPLC) and released total phenolic compounds (inhibitor) by enzymatic assay.

3.2.3 Effects of initial pH and temperature on H_2 production by an enriched hot spring culture

A hot spring sediment rich-water was collected from Beppu, Oita Prefecture, Japan, Temperature and pH at the sampling site were 94° and 7.68, respectively. Four mL of each sediment sample was enriched in 36 mL enrichment medium in a 75 mL serum bottle. Enrichment nutrient contained 10 g/L of total sugars, mixed with 4 g/L of glucose, 4 g/L of xylose and 2 g/L of arabinose, 1 g/L of yeast extract and 1 mL/L of nutrient stock solution containing 50 NH_4Cl, 30 $MgCl_2.6H_2O$, 25 KH_2PO_4, 25 K_2HPO_4, 2.5 $CoCl_2$, 2.5 $FeSO_4.7H_2O$, 1.6 $NiSO_4$, 1.5 $MnCl_2$, 1.15 $ZnCl_2$, 1.05 $CuCl_2$, and 0.5 $CaCl_2$ in g/L. The pH of the enrichment medium was adjusted to 5.5 by 3 mol/L HCl and 3 mol/L NaOH. The enrichment bottle was capped with a rubber stopper and aluminum cap, flushed with N_2 gas to obtain anaerobic conditions, and incubated at 60°C for 2 days, and then 10% of inoculum was transferred to fresh medium. Biogas, H_2, CO_2, and CH_4 were monitored during enrichment. After five cycles of subculture, the enriched culture was used as a seed inoculum in subsequent experiments.

Batch experiments were used to optimize the initial pH of the culture medium and the incubation temperature. The effect of initial pH was investigated in the range 5.5–9.0, adjusted using 3 mol/L HCl and 3 mol/L NaOH, and incubated at 60°C. The optimized pH was used to investigate the effect of incubation temperature. The incubation temperature was studied in the range 40–60°C with increments of 5.0°C. The initial substrate concentration was kept constant as in the enrichment step. The batch experiments were conducted in a 75 mL serum bottle with a working volume of 40 mL comprising 28 mL of culture medium and 12 mL of seed inoculum (140 mg/L of inoculum as measured by volatile suspended solids (VSS)). The serum bottles were fitted with rubber stoppers and aluminum caps, then flushed with N_2 gas to obtain anaerobic conditions and incubated at the desire initial pH and temperature. All experiments were conducted in triplicate. Biogas was monitored periodically.

3.2.4 H_2 production from enzymatic hydrolysate of pretreated DWC

Enzymatic hydrolysate of pretreated DWC from 1.2 was investigated as a substrate for H_2 production by the enriched hot spring culture. A batch test was conducted in a 125 mL serum bottle with a working volume of 70 mL, 21 mL (30%) of seed inoculum was centrifuged at 2,860×g for 15 min before inoculating into the hydrolysate from the enzymatic hydrolysis step containing 1 g/L of yeast extract, and 1 mL/L of nutrient stock solution. The serum bottles were fitted with rubber stoppers and aluminum caps, and then flushed with N_2 gas to obtain anaerobic conditions. The experiment was performed at the optimal pH and temperature determined from the previous study. The experiment was conducted in triplicate. Biogas was monitored periodically.

3.2.5 Analytical methods

Acid insoluble lignin was determined following the NREL method (Sluiter et al., 2011). The total phenolic compounds released during the alkaline pretreatment and enzymatic hydrolysis were analyzed by enzymatic assay (Ma and Cheung, 2007). VSS was analyzed according to a standard method.

Scanning electron microscope (SEM) observations were carried out for untreated DWC and pretreated DWC by 2% NaOH at 100°C for 60 min. Samples were simply air-dried and spread uniformly on the sample holder before images were taken.

Biogas volume was measured periodically by a wetted glass syringe method. The content of biogas in the headspace, including H_2, N_2, CH_4 and CO_2, was analyzed using a GC-8APT gas chromatography equipped with an activated carbon 60/80 mesh column and a

thermal conductivity detector (TCD) (Shimadzu, Japan). The operational temperature of the injector, column and detector were 50°C, 60°C, and 50°C, respectively. Argon was used as a carrier gas. The H_2 production potential (P) data of each test was fitted to a modified Gompertz equation (Khanal et al., 2004). The H_2 production yield (HY) and H_2 production rate (HPR) were calculated by dividing the cumulative H_2 production by the concentration of the consumed substrate and incubation time, respectively.

At the end of incubation, fermented medium was harvested and centrifuged at 7,380×g for 10 min. The supernatant was acidified with 0.2 mol/L oxalic acid for volatile fatty acids (VFAs) analysis. The concentrations of VFAs, including acetic acid (HAc), propionic acid (HPr), and butyric acid (HBu), were determined by a GC-8APF gas chromatograph equipped with a Unisole F-200 30/60 mesh packed column and flame ionization detector (FID) (Shimadzu, Japan). Nitrogen was used as a carrier gas. The operational temperature of the injector, column, and detector were 250°C, 140°C, and 250°C, respectively. Enzymatic hydrolysate and fermented medium were collected to analyze carbohydrates, including glucose, xylose and arabinose and ethanol. The concentrations of carbohydrates and ethanol were determined by HPLC (LC-10AD) equipped with a Shim-pack SPR-Pb column (Shimadzu, Japan) (oven temperature 80°C), deionized water as the mobile phase at a flow rate of 0.6 mL/min and a refraction index detector.

Microbial community of enriched hot spring culture was investigated by PCR-DGGE. Microbial sample was collected from fermented broth and kept at -20°C. Total genomic DNA of sample was extracted by using QIAamp DNA Stool Mini Kit (QIAgen, Germany). After DNA extraction, two steps of PCR amplification were conducted in this study. A universal primer pair 21f (5'-GAGTTTGATCCTTGGCTCAG-3') and 1492r (5'-GAAAGGAGGTGA TCCAGCC-3') were firstly used for amplification of approximately 1,500 bps targeting universal-consensus 16S rDNA fragment. The amplification consisted of initial denaturing step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 54°C for 40 sec, and extension at 72°C for 1 min, and final extension step at 72°C for 10 min. After that, PCR product was used as a template for second amplification, V3 region primer pair 357f with GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGG GGGGCCTACGGGAGGCGCAG-3') and 518r (5'-ATTACCGCGGCTGCTGG-3') were used as a primer for DGGE analysis. The amplification consisted of initial denaturing step at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 3 min and final extension step at 72°C for 10 min. All

PCR steps were performed in automatic thermal cycler iCyclerTM (Bio-Rad, U.S.A). The PCR-amplified rDNA fragments were assessed by DGGE by using DCode Universal Mutation Detection System (Bio-Rad, U.S.A.). Electrophoresis was conducted in 0.5X TAE buffer at 60°C, 20 volts for 20 min and followed by 70 volts for 16 hr through 8% (w/v) acrylamide gel with linear gradient denaturant ranging from 30 to 70%. The gel was stained with SYBR-Gold (1000 ng/mL) for 20 min and visualized under a UV transilluminator. The DGGE bands were excised and re-amplified with the forward primer (357f) without a GC clamp and the reverse primer (518r) under the similar reaction conditions described above. PCR products were sequenced and compared with sequences in GenBank database through BLAST search similarity.

3.3 Results and discussions

3.3.1 Lignin removal by alkaline pretreatment

The major components of DWC were classified as 47% cellulose, 16% hemicellulose, 30% lignin and 7% other components. Alkaline pretreatment was used to remove lignin from the DWC prior to the enzymatic hydrolysis step. **Figure 3.1** shows the percentage of lignin removal from DWC pretreated by alkaline pretreatment. The amount of lignin removal ranged from 9% to 41% of the initial content of lignin in the raw DWC. The results show that the maximum percentage of lignin removal was 41% when pretreated by 2% NaOH at 100°C for 60 min. Increasing the temperature (50 to 100°C) with increasing alkaline concentration (0 to 2% NaOH) improved the efficiency of delignification significantly (p-value < 0.05), and this phenomena may be affected by temperature increasing the rate of chemical reactions and mass transfer after alkaline pretreatment. However, increasing the retention time had no effect on delignification in this study; as shown by a p-value > 0.05.

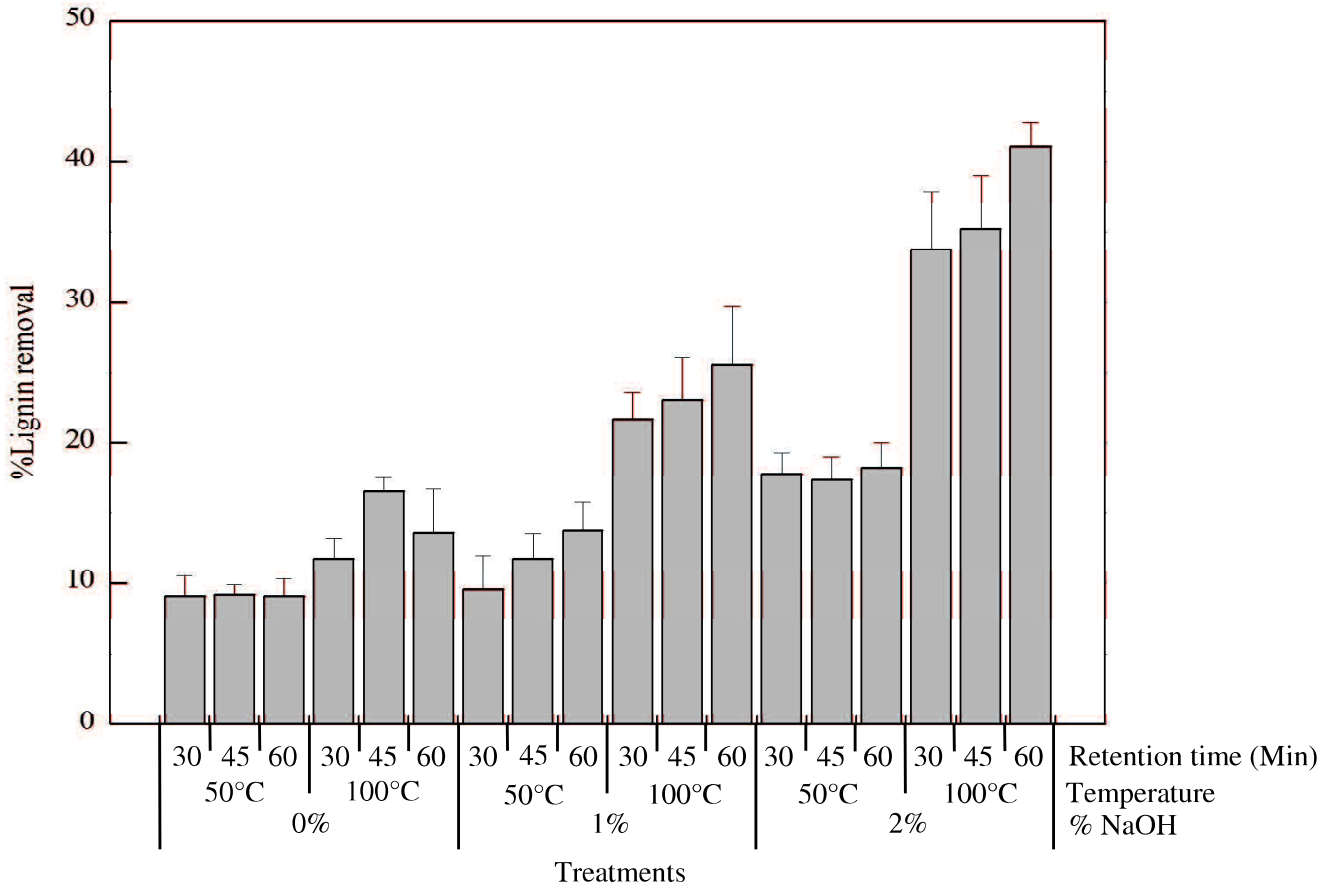

Fig. 3.1 - Percentage of lignin removal from disposable wooden chopsticks (DWC) pretreated with alkali

3.3.2 Carbohydrates released in enzymatic hydrolysate

The solid fraction of pretreated DWC was hydrolyzed by Celluclast 1.5L to obtain monosaccharides, which were easily fermented by H_2-producing bacteria. According to the percentage of lignin removal (**Fig. 3.1**), high removal corresponded to high amounts of liberated carbohydrates (**Fig. 3.2**). Glucose and xylose yields were significantly increased by increased concentrations of NaOH and increased temperature (p-value < 0.05), whereas, the arabinose yield did not change significantly with each treatment (p-value < 0.05), indicating that the release of arabinose from pretreated DWC was neither influenced by alkaline pretreatment nor enzymatic hydrolysis in this study. The highest yields of glucose and xylose were 30.65 and 29.91 mg/$g_{\text{pretreated DWC}}$, respectively, obtained from a pretreatment condition of 2% NaOH, 100°C and 60 min, and these were enhanced 23- and 5-fold released from the raw material, respectively. This may be an evidence that alkaline pretreatment degraded lignin mainly from the cellulosic biomass, and this allowed enzyme to obtain easier access to cellulose molecules during enzymatic hydrolysis (Taherzadeh and Karimi, 2008). Moreover, xylose could be liberated by Celluclast 1.5L, indicated that it may also harbor a β-xylosidase

activity, capable of catalyzing the hydrolysis of short chain xylooligomers, including xylobiose and xylotriose, to xylose (Sørensen et al., 2005).

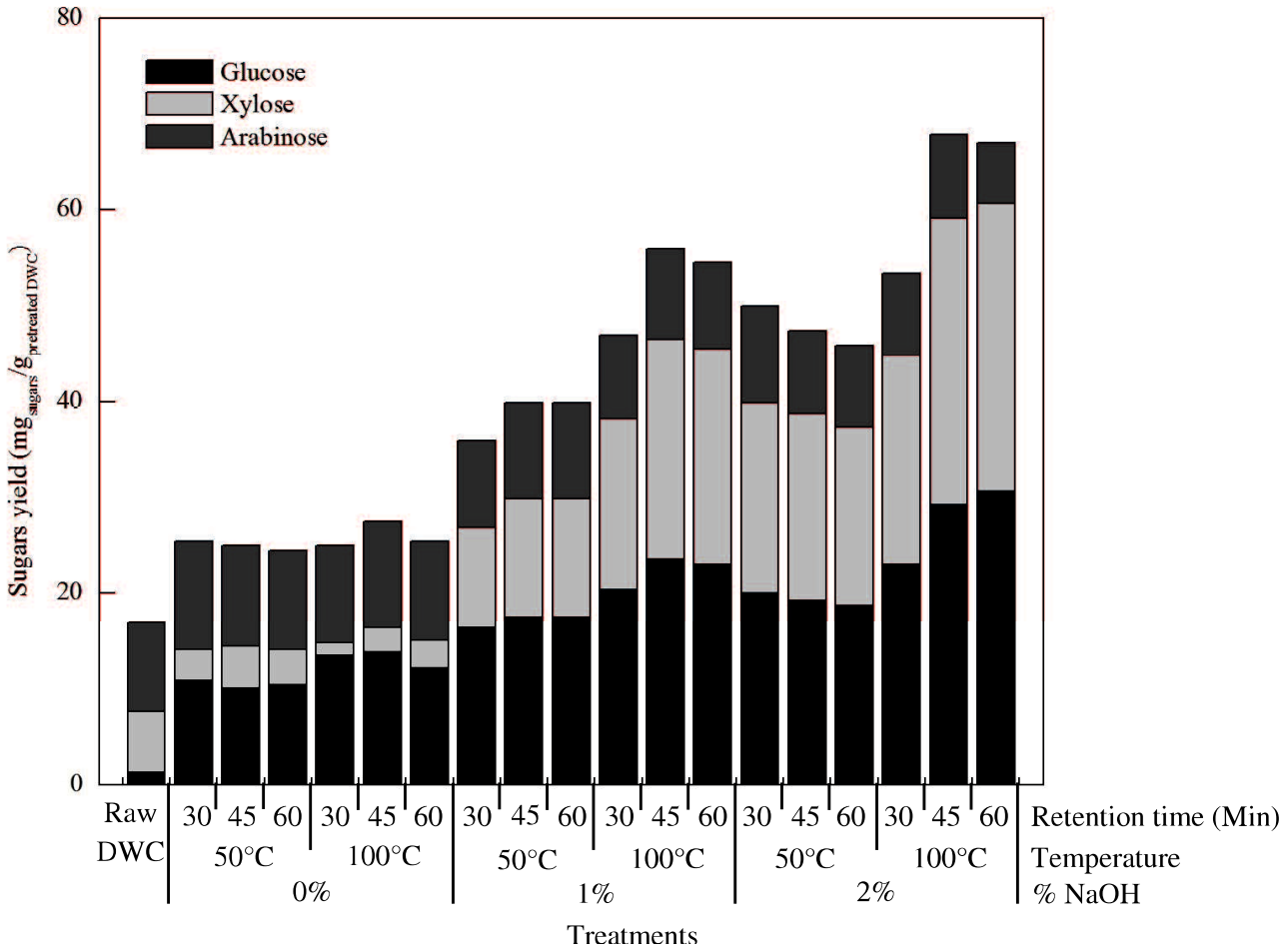

Fig. 3.2 - Monosaccharide composition of carbohydrates released from pretreated and enzymatically saccharified DWC

3.3.3 Phenolic compounds released from pretreatment and enzymatic hydrolysis steps

Phenolic compounds have been reported extensively in the literature as being released into the liquid fraction during pretreatment of lignocellulosic materials (Klinke et al., 2002; Du et al., 2010). These compounds were identified as an inhibitor that affects downstream processes, including enzymatic hydrolysis and fermentation (Klinke et al., 2004). Therefore, it was necessary to monitor the amount of phenolic compounds released into the liquid fraction after pretreatment and enzymatic hydrolysis steps, as shown in **Fig. 3.3**. Phenolic compounds release into the liquid fraction was dramatically higher under severe alkaline pretreatments (high concentration of NaOH and high temperature, at 100°C). Most of the phenolics were released into the pretreatment liquid phase (maximum of 993 ng/g), so that the total phenolics content in the enzyme hydrolysate was much lower (maximum of 85 ng/g).

These results suggest that harsher pretreatment conditions can remove most of the phenolic content during the pretreatment step and reduce the content of phenolic compounds in the enzyme hydrolysate. Therefore, detoxification was not required after pretreatment; hydrolysate could be used directly as a feedstock for H_2 production in this study.

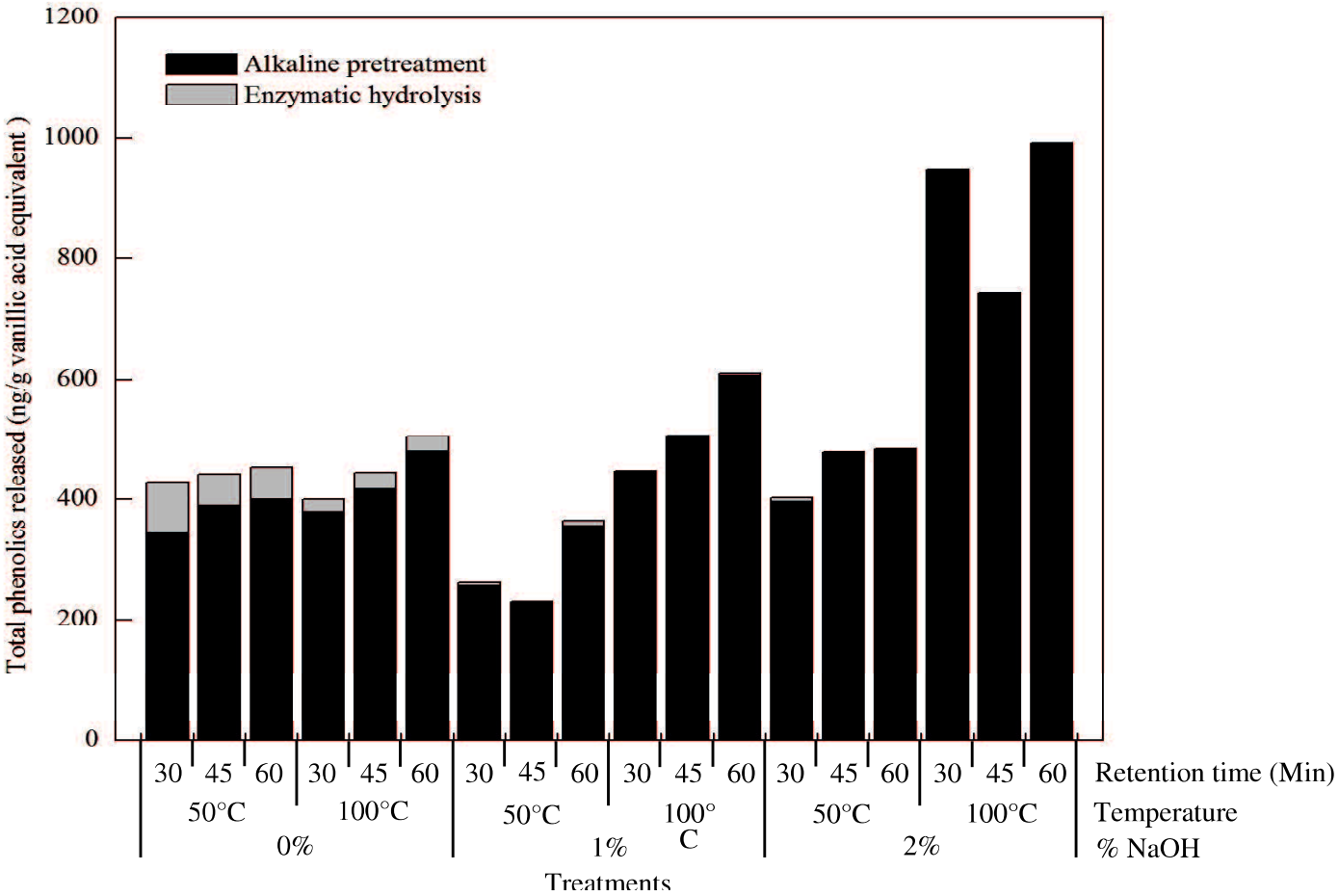

Fig. 3.3 - Total phenolics released during alkaline pretreatment and enzymatic hydrolysis steps

3.3.4 SEM observations

SEM observation were purposed to examine the physical structural changes in the biomass after alkaline pretreatment. SEM images of untreated and pretreated DWC were revealed in **Fig. 3.4**. Untreated DWC had a relatively smooth and continuous surface structure (**Fig. 3.4a**) whereas surface significant structure changes occurred after pretreatment (**Fig. 3.4b**). This could be evidence that alkaline pretreatment mainly degraded lignin from cellulosic biomass, and this allowed enzyme to get easier access to cellulose molecules during enzymatic hydrolysis. Moreover the surface of the pretreated DWC was corroded and many small pores were observed after enzymatic hydrolysis (**Fig. 3.4c**).

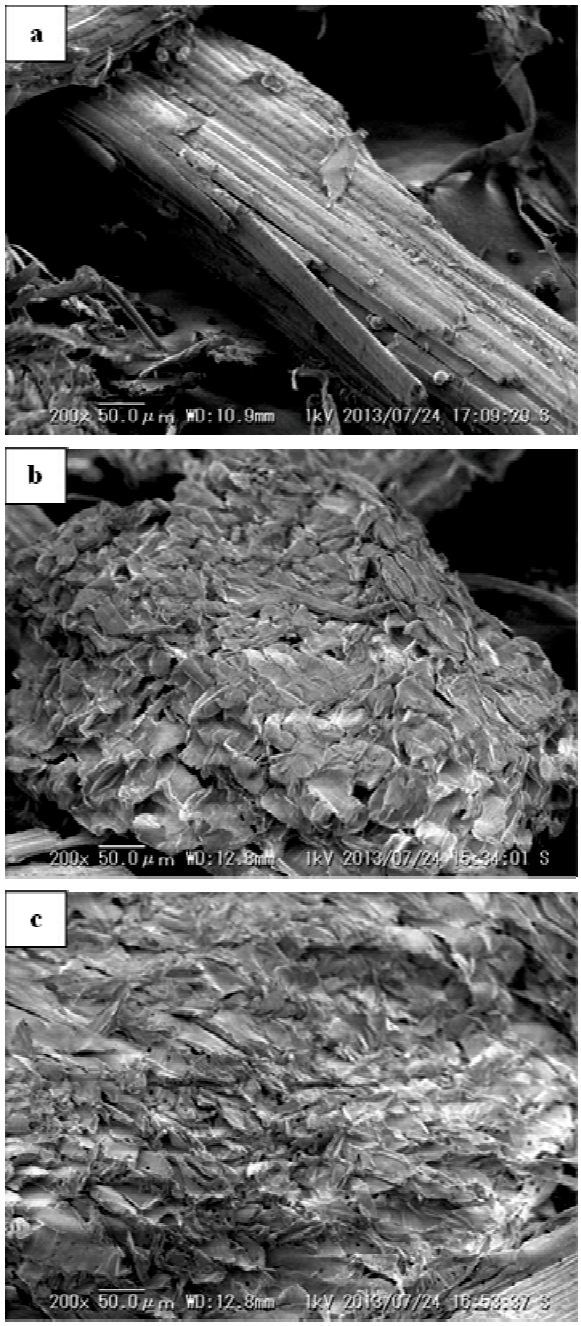

Fig. 3.4 - SEM images of untreated DWC (a); pretreated DMC at 2% NaOH, 100°C and 60 min (b) and pretreated DWC after enzymatic hydrolysis (c)

3.3.5 Effects of initial pH and temperature on H_2 production by the enriched hot spring culture

The effect of initial pH on H_2 production was studied over the range 5.5–9.0 (**Fig. 3.5**). The experiment was also set up at pH 5.0 but unfortunately there was no H_2 production obtained (data not shown). Similarly, Puhakka et al. (2012) also revealed that no hydrogen was generated at the initial pH 5.0 from glucose by enriched hot spring mesophilic and thermophilic consortia. The maximum HY and HPR obtained were 187 mL H_2/g total $sugar_{consumed}$ and 141 mL H_2/L.day, respectively, at an initial pH of 7.0 (**Fig. 3.5a**), where the optimal pH value was similar to that of sediment-rich water in the hot spring. This finding may indicate a microorganism habitat preference. Zhang et al. (2007) have also reported that an initial pH of 7.0 was optimal for H_2 production from cornstalk wastes by mixed anaerobic cultures. Therefore, this optimal pH was used subsequently to investigate the effect of temperature on H_2 production.

The effect of temperature on H_2 production was investigated over the range 40–60°C (**Fig. 3.6**). The results show that HY and HPR were correlated at all temperatures, with maximum values obtained of 173 mL H_2/g total $sugar_{consumed}$ and 190 mL H_2/L.day, respectively, at 50°C (**Fig. 3.6a**). At lower temperatures, 40–45°C, H_2 production rates were significantly lower because the lag phase of the microorganisms (69–166 hr) was longer than that at higher temperature, 50-60°C (13–29 hr) (data not shown). The experiment was also tested at 65°C but H_2 could not be produced at this temperature (data not shown). These findings support the conclusion that the productivity of H_2-producing bacteria can be enhanced by increasing temperature. However, temperature can decrease H_2 production when increased beyond a certain point (Wang and Wan, 2008).

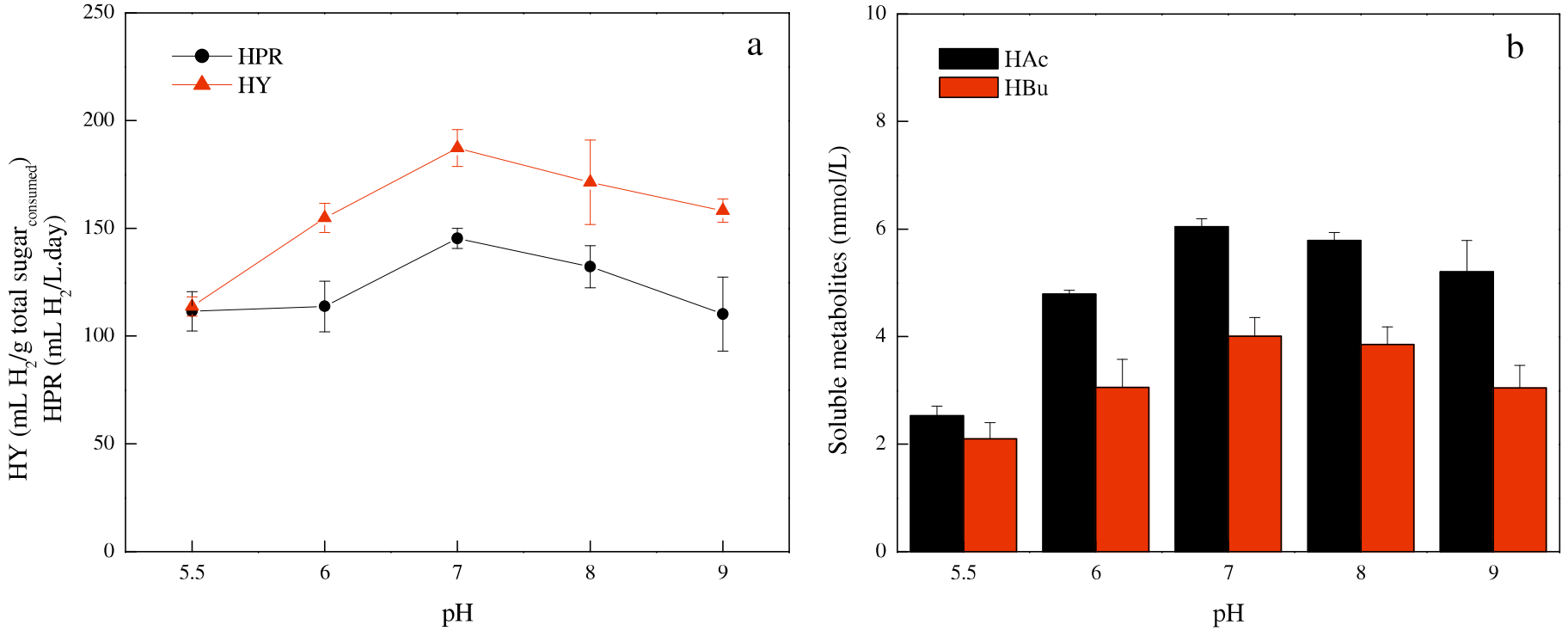

Fig. 3.5 - The effect of initial pH on H_2 production (a) and soluble metabolites (b) by the enriched hot spring culture; HPR: H_2 production rate; HY: H_2 yield; HAc: acetic acid; HBu: butyric acid

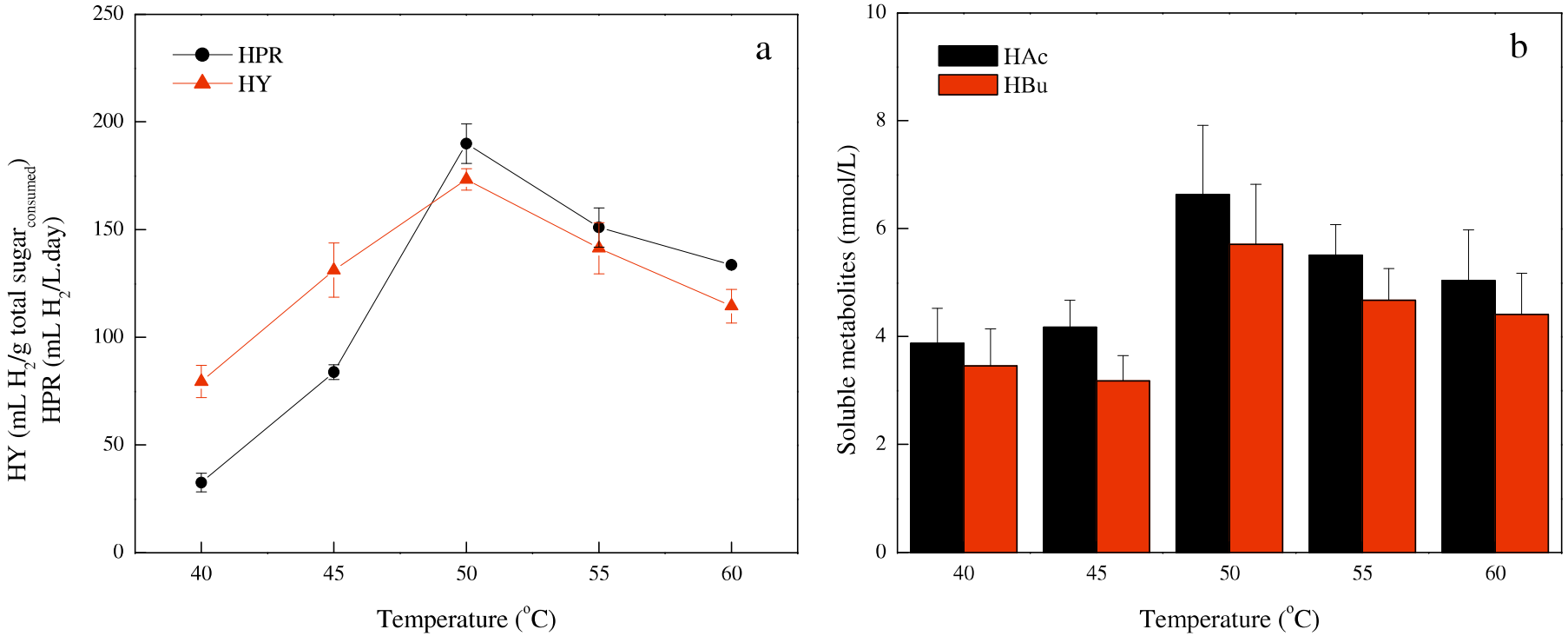

Fig. 3.6 - The effect of temperature on H_2 production (a) and soluble metabolites (b) by the enriched hot spring culture; HPR: H_2 production rate; HY: H2 yield; HAc: acetic acid; HBu: butyric acid

At the end of fermentation, the pH had decreased because soluble metabolites were released into the culture medium. In **Figs. 3.5b and 3.6b**, acetic and butyric acids were observed at levels of approximately 2.53–6.63 mmol/L and 2.10–5.71 mmol/L, respectively. Thus, it could be concluded that acetate-butyrate fermentation occurred during H_2 production by the enriched hot spring culture used in this study. In addition, no methane was observed throughout the experiments, even when no pretreatment was conducted prior to the enrichment of the sediment-rich water from the hot spring.

3.3.6 H_2 production from the enzymatic hydrolysate of pretreated DWC

To demonstrate the feasibility of using DWC as a feedstock for H_2 production, the enzymatic hydrolysate from the best conditions from the alkaline pretreatment step, containing 1.23 g/L glucose, 2.47 g/L xylose and 0.54 g/L arabinose, was applied as a substrate for H_2 production (**Table 3.1**). Cumulative H_2 was calculated using the modified Gompertz equation at 564 mL/L, while HY and HPR were observed at 195 mL H_2/g total $sugars_{consumed}$ and 116 mL H_2/L.day, respectively. Glucose was completely consumed during fermentation, followed by 74% arabinose and 42% xylose, respectively. Fermentative H_2 production was accompanied by the formation of the soluble metabolites presented in **Table 3.1**. Acetic and butyric acids were the major metabolites of the hot spring enriched culture. Other metabolites such as formic acid, lactic acid, acetone and butanol may be produced during the fermentation but could not be detected because of the limitation of the analytical equipment.

Table 3.1 - Performance of fermentative H_2 production by the enriched hot spring culture using enzymatic hydrolysate of pretreated DWC as a substrate.

Cumulative H_2 (mL H_2/L)	HY (mL H_2/ g total $sugar_{consumed}$)	HPR (mL H_2/ L.day)	Glucose (g/L)		Xylose (g/L)		Arabinose (g/L)		Total Sugars (g/L)		Soluble Metabolites (mmol/L)		
			Initial	Final	Initial	Final	Initial	Final	Initial	Final	HAc	HPr	HBu
564	195	116	1.23	0.00 (100)[a]	2.47	1.14 (42)	0.54	0.14 (74)	4.24	1.28 (70)	6.14	0.14	3.33

[a]: percentage of substrate consumption; HY: H_2 yield; HPR: H_2 production rate; HAc: acetic acid; HPr: Propionic acid; HBu: butyric acid

3.3.7 Microbial community

PCR-DGGE was applied for determination of microbial community obtained from enriched hot spring culture. **Figure 3.7** illustrated DGGE profile of the 16S rDNA gene fragments obtained from microbial sample collected at the end of fermentation from Section 2.5. The major bands in the DGGE gels were excised and determined the sequences. The results of the sequence affiliation determined by the BLAST were shown in **Table 3.2**. Band 1 and 2 were affiliated with *D. incerta* and *Pisciglobus* sp., respectively. Both strains have been identified as lactic acid producers from glucose without H_2 production (Stackebrandt et al., 1999 and Tanasupawat, et al., 2011). These strains are possibly coincided as substrate competitors of H_2-producing bacteria. *Thermoanaerobacterium thermosaccharolyticum*, *Thermoanaerobacterium xylanolyticum* and *Thermoanaerobacterium* sp. were affiliated with Band 3, 4 and 6, respectively. *Thermoanaerobacterium* spp. are well known for its characteristics of H_2 production and utilization of a variety of monosaccharaides including glucose, xylose and arabinose in the previous studies (Ren et al., 2008; Khamtib and Reungsang, 2012 and Saripan and Reungsang, 2013). *Bacillus* sp. was also detected in the microbial culture (Band 5), some species of Bacillus genus have been reported for the capability of H_2 production (Patel et al., 2011 and Kotay and Das, 2007).

Table 3.2 - Affiliation of DGGE fragments determined by their 16S rDNA sequences

Band	Microorganism	%Identity	Accession No.
1	*Pisciglobus* sp. SIF5	100	KF186669.1
2	*Desemzia incerta* strain SB-B1	100	KC577171.1
3	*Thermoanaerobacterium thermosaccharolyticum* DSM 571	99	NR_074419.1
4	*Thermoanaerobacterium xylanolyticum* LX-11	97	NR_102771.1
5	*Bacillus* sp. Rai12	100	AB845215.1
6	*Thermoanaerobacterium* sp. K162C	99	HQ840649.2

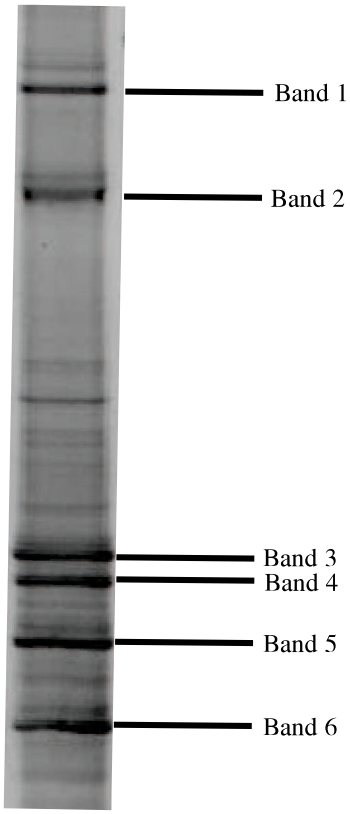

Fig. 3.7 - DGGE profile of 16S rDNA gene fragments extracted from the enriched hot spring culture

3.4 Conclusions

The feasibility of using DWC as a feedstock for fermentative H_2 production was established in this study. Alkaline pretreatment was conducted for delignification prior to enzymatic hydrolysis, and 2% (w/v) NaOH at 100°C was the most effective treatment according to the highest percentage of lignin removal and the concentrations of carbohydrates released. In addition, the optimal conditions for the enriched hot spring culture were determined and an initial pH 7.0 culture medium incubated at 50°C attained the highest H_2 production. Furthermore, enzymatic hydrolysate from pretreated DWC was successfully demonstrated as a substrate for fermentative H_2 production, with HY and HPR being achieved at 195 mL H_2/g total $sugars_{consumed}$ and 116 mL H_2/L.day, respectively. *Thermoanaerobacterium* spp. are the H_2-producing bacteria mainly present in the fermentation process. The results in this study show a potential application for converting DWC into fermentable carbohydrates and H_2 energy even

though the conversions of cellulose and hemicellulose into fermentable carbohydrates were low in the hydrolysate obtained from enzymatic hydrolysis step. Thus more effort is needed to develop, for example, the enzymatic hydrolysis step to enhance the released carbohydrate yield and to optimize the culture media to improve the efficiency of H_2-producing bacteria to increase H_2 production.

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CHAPTER IV

OPTIMIZATION OF ENZYMATIC HYDROLYSIS FOR PRETREATED WOOD WASTE BY RESPONSE SURFACE METHODOLOGY IN FERMENTATIVE HYDROGEN PRODUCTION

4.1 Introduction

Recently, lignocellulosic biomass such as agricultural and forest residue has become an interesting potential resource and serves as a renewable feedstock for fermentative H_2 production, due to its costless. The waste from disposable wooden chopsticks (DWC) was represented as wood waste and investigated in this study. DWC are used daily in Asian countries such as China, Taiwan, Korean and Japan, with an annual consumption of approximately 25 billion pairs (90,000 tons) in Japan made from aspen and white birch wood (Asada et al., 2011). There is significant potential for producing H_2 from DWC to turn waste into energy. Lignocellulosic biomass cannot be fermented directly by most of H_2 producing bacteria, and thus, it needs to be converted into fermentable sugar. The three main processes of fermentative H_2 production from lignocellulosic biomass are: pretreatment, hydrolysis, and fermentation (Wi et al., 2011). Acid and enzymatic hydrolysis is generally applied to convert pretreated biomass into fermentable sugar. However, enzymatic hydrolysis appears to be a better technology in terms of being neither an inhibitor nor byproduct which might have an effect on the downstream processes (El-Zawawy et al.,2011). Enzymatic hydrolysis was affected by several factors, such as biomass and enzyme loading, surfactant concentration, and hydrolysis times. The cellulase enzyme is commonly introduced in enzymatic hydrolysis of lignocellulosic biomass and, consisting of three groups of enzymes: (1) endoglucanase, which randomly hydrolyzes regions of low crystallinity in the cellulose fiber and produces free chain-ends; (2) exoglucanase, which cleaves at the free chain-ends liberating soluble glucose or cellobiose; and (3) β-glucosidase, which hydrolyzes cellobiose to produce glucose and could prevent cellobiose inhibition in the process (Zhang et al., 2006). Currently, most commercial cellulases were produced by the Trichoderma species but were lacking in the production of β-glucosidase. Thus, the addition of β-glucosidase was required to completely hydrolyze cellulose into glucose (Ahamed and Vermette 2008a). Several studies have reported that the addition of non-ionic surfactants could improve the enzymatic hydrolysis of pretreated biomass or reduce the amount of enzyme loading (Eriksson et al., 2002; Kristensen

et al., 2007; Qing et al., 2010; Tu and Saddler, 2010). Since there are multiple factors affecting enzymatic hydrolysis, response surface methodology (RSM) was used to design the experiment and determine the optimal conditions for the desirable responses. This method has many advantages over the conventional method (one factor at a time) in terms of time saving and reduction in the work required, together with the opportunity to examine the interaction amongst variables (Bezerra et al., 2008). RSM has been successfully applied for the optimization of enzymatic hydrolysis of various lignocellulosic biomass e.g., rice straw (Singh and Bishnoi, 2012a); wheat straw (Singh and Bishnoi, 2012b); aspen (Jagtap, et al., 2013) and sugarcane top (Sindhu et al., 2014). So far, this method has been used for optimizing enzymatic hydrolysis in most agricultural residues; however, there have been small studies on the optimization of enzymatic hydrolysis of wooden material. The objective of this work is to optimize the enzymatic hydrolysis condition of pretreated DWC by RSM for using as a substrate for fermentative H_2 production.

4.2 Materials and methods

4.2.1 Raw material

DWC waste was collected from the cafeteria at the Faculty of Science and Engineering, Yamaguchi University, Yamaguchi Prefecture, Japan. DWC were soaked in tap water overnight and washed with detergent, then dried at 45°C overnight. Dried DWC were ground in a mill (LM-Plus, Osaka Chemical, Osaka, Japan) and passed through a 2 mm screen, then stored at room temperature in a sealed container.

4.2.2 Alkaline pretreatment

10% (w/v dry mass) of DWC was pretreated with 2% NaOH at 100°C for 30 minutes (Phummala et al., 2014). After that, pretreated DWC (cellulose fractions) were sieved through a muslin cloth, and washed with tap water until neutral, dried at 45°C, and stored at room temperature in a sealed container.

4.2.3 Enzymatic hydrolysis

Enzymatic hydrolysis of alkaline pretreated DWC was conducted in a test tube containing 50 mM citrate buffer (pH 4.8), 10 mM sodium azide to prevent microbial contaminant growth. Cellulase (Celluclast 1.5L) and β-glucosidase (Novozyme 188) were purchased from Sigma Chemicals, St. Louis, MO, USA. The mixture was incubated at 50°C. At the end of incubation, samples were immediately heated to 100°C to denature the enzymes,

cooled, and then centrifuged for 10 minutes at 7,380×g. The supernatant was analyzed for concentrations of glucose and reducing sugar.

4.2.4 Experiment design for enzymatic hydrolysis

4.2.4.1 Plackett-Burman design (PBD)

PBD was used to identify the factors affecting the enzymatic hydrolysis significantly. Five parameters; solid loading (w/v dry mass) cellulase dosage (FPU/g pretreated DWC), β-glucosidase dosage (CBU/g pretreated DWC), Tween 80 (g/g pretreated DWC), and hydrolysis time (h), were investigated in the present study. The level of each parameter was set at two levels: -1 for low and +1 for high as shown in **Table 4.1**. The experiment design matrix is presented in **Table 4.2**.

Table 4.1 - Levels of experimental variables, estimated effect, and *p*-values in PBD

Variables	Unit	Symbol code	Levels		Glucose yield[a]		Reducing sugar yield[b]	
			-1	+1	Effect (E_{xi})	*p*-value	Effect (E_{xi})	*p*-value
Solid loading	% (w/v)	X_1	5	10	-1.18	0.6584	-1.11	0.8924
Cellulase dosage	FPU/g pretreated DWC	X_2	5	10	15.70	0.0008[c]	42.64	0.0016[d]
β-glucosidase dosage	CBU/g pretreated DWC	X_3	10	20	6.87	0.0353[c]	21.42	0.0340[d]
Tween 80	g/g pretreated DWC	X_4	0.02	0.1	5.26	0.0838[c]	12.17	0.1713
Hydrolysis time	h	X_5	24	72	19.36	0.0003[c]	44.97	0.0012[d]

[a] $R^2 = 0.9474$; adj $R^2 = 0.9036$

[b] $R^2 = 0.9236$; adj $R^2 = 0.8599$

[c] and [d] *p*-values <0.1 (Significant for 90% confidence level) for glucose and reducing sugar yield, respectively.

4.2.4.2 Enzymatic hydrolysis by response surface methodology (RSM)

In order to optimize the level of significant variables of enzymatic hydrolysis; central composite design (CCD) was adopted in this study. The significant variables were screened based on the results of PBD as follows: cellulase dosage, β-glucosidase dosage, Tween 80, and hydrolysis time, and were assessed at five coded levels (-2, -1, 0, +1, +2). The levels of variables and the experimental design are shown in **Table 4.3**. A total of 30 experiments were

conducted during this step. The response values, glucose, and reducing sugar yields, are an average of the triplicates. The quadratic model for predicting the response was expressed as an equation (4.1).

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (4.1)$$

where Y is the predicted response (Sugar yield); β_0 is a constant; β_i is the linear coefficient; β_{ii} is the squared coefficient; β_{ij} is the interaction coefficient; x_i is the variable i, and x_j is the variable j. The response variable was fitted using a predictive polynomial quadratic equation (Eq. (4.1)) in order to correlate the response variable to the independent variables. The statistical software Design-Expert® (Trial version 9.0, Stat-Ease, Inc., Minneapolis, MN, USA) was used for regression and graphical analysis of the experimental data.

4.2.5 H_2 production

An anaerobic mixed culture was enriched from a hot spring; optimal pH and temperature was investigated at 7.0 and 50°C, respectively (Phummala et al., 2014). The mixed culture was used as an inoculum for fermentative H_2 production. The inoculum was cultured in a medium containing 5 g/L each of glucose and xylose, 1 g/L of yeast extract, 0.5 g/L of L-cysteine, 2.6 g/L $NaHCO_3$ and 1 mL/L of nutrient stock solution containing 300 $MgCl_2.6H_2O$, 250 KH_2PO_4, 250 K_2HPO_4, 25 $CoCl_2.6H_2O$, 25 $FeSO_4.7H_2O$, 16 $NiCl_2.6H_2O$, 15 $MnCl_2.4H_2O$, 11.5 $ZnCl_2$, 10.5 $CuCl_2$, and 5 $CaCl_2$ in g/L for 24 h. A batch test was conducted in a 30 mL serum bottle with a working volume of 15 mL. 30% of inoculum was centrifuged at 2,860×g for 15 minutes before inoculation into the enzymatic hydrolysate supplement with 1 g/L of yeast extract, 0.5 g/L of L-cysteine, 2.6 g/L $NaHCO_3$ and 1 mL/L of nutrient stock. The serum bottles were fitted with rubber stoppers and aluminum caps, and then flushed with N_2 gas to obtain anaerobic conditions. The experiment was conducted in triplicate at the optimal pH and temperature, as mentioned above. Biogas was monitored periodically until none was produced.

4.2.6 Analytical methods.

Enzymatic hydrolysate and fermented medium were collected to analyze glucose, xylose and ethanol. The concentrations of glucose, xylose and ethanol were determined by HPLC (Phummala et al., 2014). Reducing sugar was analyzed by the dinitrosalicylic acid (DNS) method. Biogas volume was measured periodically by using a wet glass syringe. The

content of biogas in the headspace, including H_2, N_2, CH_4 and CO_2, was analyzed using a GC-8APT gas chromatograph, equipped with a thermal conductivity detector (TCD) (Shimadzu, Kyoto, Japan) (Phummala et al., 2014). The cumulative H_2 production data of each test was fitted to a modified Gompertz equation (Khanal et al., 2004). At the end of incubation, fermented medium was harvested and centrifuged at 7,380×g for 10 minutes. The supernatant was acidified with 0.2 N oxalic acid for volatile fatty acids (VFAs) analysis. The concentrations of VFAs, including acetic acid (HAc), propionic acid (HPr), and butyric acid (HBu), were determined by a GC-8APF gas chromatograph equipped flame ionization detector (FID) (Shimadzu, Kyoto, Japan) (Phummala et al., 2014).

4.3 Results and discussions

4.3.1 Screening factors affecting enzymatic hydrolysis by PBD

PBD is a powerful tool used to identify significant factors and the emphasis of those factors on further steps in the optimization process. A total of five factors were investigated to discover their effect on enzymatic hydrolysis, the level of each factor, estimated effect, and probability value (*p*-value) on corresponding sugar yields as presented in **Table 4.1**. The design matrix and the corresponding responses, glucose and reducing sugar yields, are shown in **Table 4.2**. The results indicate that the effect of cellulase dosage, β-glucosidase dosage, Tween 80, and hydrolysis time, were greater at a high level and showed as having a positive effect on glucose and reducing sugar yields. On the other hand, the percentage of solid loading showed a negative effect on those responses. Factors having a confidence greater than 90% (*p*-value < 0.1) were considered to have a significant effect on glucose and reducing sugar yields. The percentage of solid loading showed confidence levels below 90% and were considered insignificant on glucose and reducing sugar yields. Cellulase dosage, β-glucosidase dosage, and hydrolysis time were found to have a significant effect on both glucose and reducing sugar yields. Nevertheless, Tween 80 was found to significantly influence glucose yields individually. Therefore, cellulase dosage, β-glucosidase dosage, Tween 80, and hydrolysis time, were selected for a further optimization study.

4.3.2 Optimization of enzymatic hydrolysis by CCD

The effect of four variables; cellulase dosage (X_2), β-glucosidase dosage (X_3), Tween 80 (X_4), and hydrolysis time (X_5), were further investigated for the optimal conditions of enzymatic hydrolysis using CCD. While the percentage of solid loading was found insignificant in the screening procedure with a negative effect, this variable was kept to a low

level at 5% (w/v) of pretreated DWC. The design matrix, with the experimental and predicted results is presented in **Table 4.3**. According to the data from **Table 4.3**, the corresponding responses, glucose and reducing sugar yields, were fitted with the second-order polynomial equations as shown as Eq. (4.2) and Eq. (4.3), respectively.

$$\text{Glucose Yield} = 36.54 + 2.29X_2 + 1.63X_3 - 26.99X_4 + 0.18X_5 + 0.0074X_2X_3 + 0.41X_2X_4 - 0.0053X_2X_5 + 0.58X_3X_4 + 0.0036X_3X_5 + 0.62X_4X_5 - 0.032X_2^2 - 0.025X_3^2 - 145.93X_4^2 - 0.0016X_5^2 \quad (4.2)$$

$$\text{Reducing Sugar Yield} = -1.70 + 7.07X_2 + 6.70X_3 - 5.28X_4 + 2.34X_5 - 0.0058X_2X_3 - 2.18X_2X_4 + 0.0013X_2X_5 + 8.00X_3X_4 - 0.023X_3X_5 - 1.60X_4X_5 - 0.080X_2^2 - 0.063X_3^2 - 87.68X_4^2 - 0.0045X_5^2 \quad (4.3)$$

where X_2 is the cellulase dosage, X_3 is β-glucosidase dosage, X_4 is Tween 80 and X_5 is the hydrolysis time.

The results of ANOVA for the regression model are shown in **Table 4.4**. The probability values (*p*-value) of the regression model were less than 0.0001, with an insignificant lack-of fit ($p>0.1$) for both responses, indicating that the models are significant. The coefficients of determination (R^2) were examined at 0.9207 and 0.9208 for glucose yield and reducing sugar yield models respectively, indicating 92% of variability in both models. In addition, adjusted coefficients of determination (Adj. R^2) indicated that the accuracy and general availability of the polynomial model were adequate in both models.

Table 4.2 - PBD matrix with sugar yields as responses

Run	Factors					Responses (mg/g pretreated DWC)	
	X_1	X_2	X_3	X_4	X_5	Glucose yield	Reducing Sugar yield
1	10	5	20	0.10	72	78.13±12.92	249.65±4.60
2	10	10	10	0.10	72	83.39±1.18	281.65±1.77
3	5	5	10	0.02	24	45.05±0.95	176.20±7.07
4	5	5	10	0.02	72	68.50±1.96	231.20±3.77
5	10	10	20	0.02	24	73.21±2.04	257.65±10.69
6	5	10	20	0.02	72	87.36±0.33	279.87±5.66
7	10	5	20	0.02	72	65.53±6.51	231.90±12.02
8	5	10	10	0.10	72	86.23±9.97	270.87±7.07
9	10	10	10	0.02	24	55.65±0.26	196.90±10.61
10	5	5	20	0.10	24	55.15±3.13	204.20±0.47
11	5	10	20	0.10	24	72.33±4.72	251.20±32.06
12	10	5	10	0.10	24	51.63±0.48	189.15±4.60

Table 4.3 - Experimental design and results of CCD

Run	Variables				Responses			
	X_2: Cellulase dosage (FPU/g pretreated DWC)	X_3: β-glucosidase dosage (CBU/g pretreated DWC)	X_4: Tween 80 (g/g pretreated DWC)	X_5: Hydrolysis time (h)	Glucose yield (mg/g pretreated DWC)		Reducing sugar yield (mg/g pretreated DWC)	
					Experimental	Predicted	Experimental	Predicted
1	7.5	42.5	0.3	102	98.3±3.3	99.8	345.2±40.5	345.7
2	45	55	0.2	132	110.8±7.2	112.1	400.0±46.2	406.5
3	57.5	42.5	0.3	102	104.3±13.0	103.5	400.9±12.7	402.7
4	20	30	0.2	72	107.9±6.1	109.1	357.0±49.2	355.4
5	20	30	0.4	72	100.3±2.5	99.8	365.2±32.5	362.4
6	45	55	0.4	132	120.8±8.4	118.2	430.3±10.4	425.9
7	45	30	0.2	72	110.3±8.8	111.3	376.9±36.3	391.5
8	45	55	0.2	72	117.1±9.5	117.0	410.1±36.6	407.5
9	32.5	42.5	0.3	102	123.4±4.7	121.8	430.2±50.4	424.3
10	20	55	0.2	72	110.7±12.0	110.1	374.5±12.1	375.0
11	32.5	42.5	0.3	102	122.7±0.0	121.8	408.2±6.1	424.3
12	32.5	17.5	0.3	102	99.0±7.1	98.6	360.5±17.5	359.1
13	32.5	42.5	0.3	102	124.9±9.6	121.8	425.2±4.8	424.3
14	20	30	0.2	132	107.0±1.3	106.7	377.9±7.5	386.6
15	20	55	0.4	72	105.1±3.8	104.4	422.8±44.3	431.9
16	32.5	42.5	0.03	102	115.0±22.5	114.3	410.9±13.7	405.7
17	32.5	42.5	0.53	102	109.7±8.4	111.1	424.5±28.0	432.0
18	45	55	0.4	72	112.8±7.7	113.9	455.9±24.5	450.8
19	32.5	42.5	0.3	102	117.6±8.0	121.8	442.9±9.9	424.3
20	45	30	0.2	132	101.7±5.8	100.9	439.8±12.5	424.6
21	20	55	0.2	132	114.2±8.6	113.2	375.0±21.2	372.1
22	32.5	42.5	0.3	102	114.7±1.4	121.8	416.2±14.1	424.3
23	20	55	0.4	132	117.1±2.9	116.8	416.2±1.7	405.2
24	45	30	0.4	132	102.2±3.6	103.5	390.8±54.2	394.0
25	32.5	67.5	0.3	102	113.3±9.9	114.4	407.0±21.5	410.7
26	32.5	42.5	0.3	162	115.7±6.6	117.2	403.2±36.1	411.2
27	45	30	0.4	72	105.0±8.7	104.6	388.0±6.5	384.9
28	20	30	0.4	132	108.0±15.1	106.7	373.0±24.3	369.6
29	32.5	42.5	0.3	102	127.5±12.6	121.8	423.4±4.0	424.3
30	32.5	42.5	0.3	42	116.1±0.7	115.3	410.6±1.7	404.9

Table 4.4 - ANOVA of CCD for glucose and reducing sugar yield.

Glucose Yield					
Source	Sum of Squares	DF	Mean Square	F-Value	*p*-value
Model	1643.78	14	117.41	12.43	< 0.0001
Residual	141.64	15	9.44		
Lack of Fit	27.35	10	2.73	0.12	0.9976
Pure Error	114.29	5	22.86		
Cor Total	1785.42	29			
R-Squared	0.9207				
Adj R-Squared	0.8466				
Reducing Sugar Yield					
Source	Sum of Squares	DF	Mean Square	F-Value	*p*-value
Model	20632	14	1473.71	12.46	< 0.0001
Residual	1774.63	15	118.31		
Lack of Fit	1067.95	10	106.79	0.76	0.6707
Pure Error	706.69	5	141.34		
Cor Total	22406.64	29			
R-Squared	0.9208				
Adj R-Squared	0.8469				

Figures 4.1 and **4.2** present the significant interaction effect of variables on the glucose yield and reducing sugar yield, respectively. The plots were illustrated by varying two variables within the experimental range, while two other variables were kept constant at the center point. The interaction effect between cellulase dosage and hydrolysis time on glucose yield is shown in **Fig. 4.1a**. Glucose yield increased with cellulase dosage of up to 36 FPU/g pretreated DWC. Beyond that level, glucose yield was reduced. This may be caused by the timing of product accumulation interval hydrolysis leading to inhibition of cellulase activity. **Figures 4.1b** and **4.2b** are presented to show the interaction between β-glucosidase dosage and hydrolysis time on glucose and reducing sugar yields, respectively. Sugar yields were increased, corresponding to an increase in β-glucosidase dosage and hydrolysis time. Since Celluclast 1.5L is produced from *T. reesei*, it contains low β-glucosidase activity (Ahamed and Vermette, 2008b). Cellobiose, an end product of exoglucanase, may be incompletely hydrolyzed and accumulate in the hydrolysate. This may explain why a

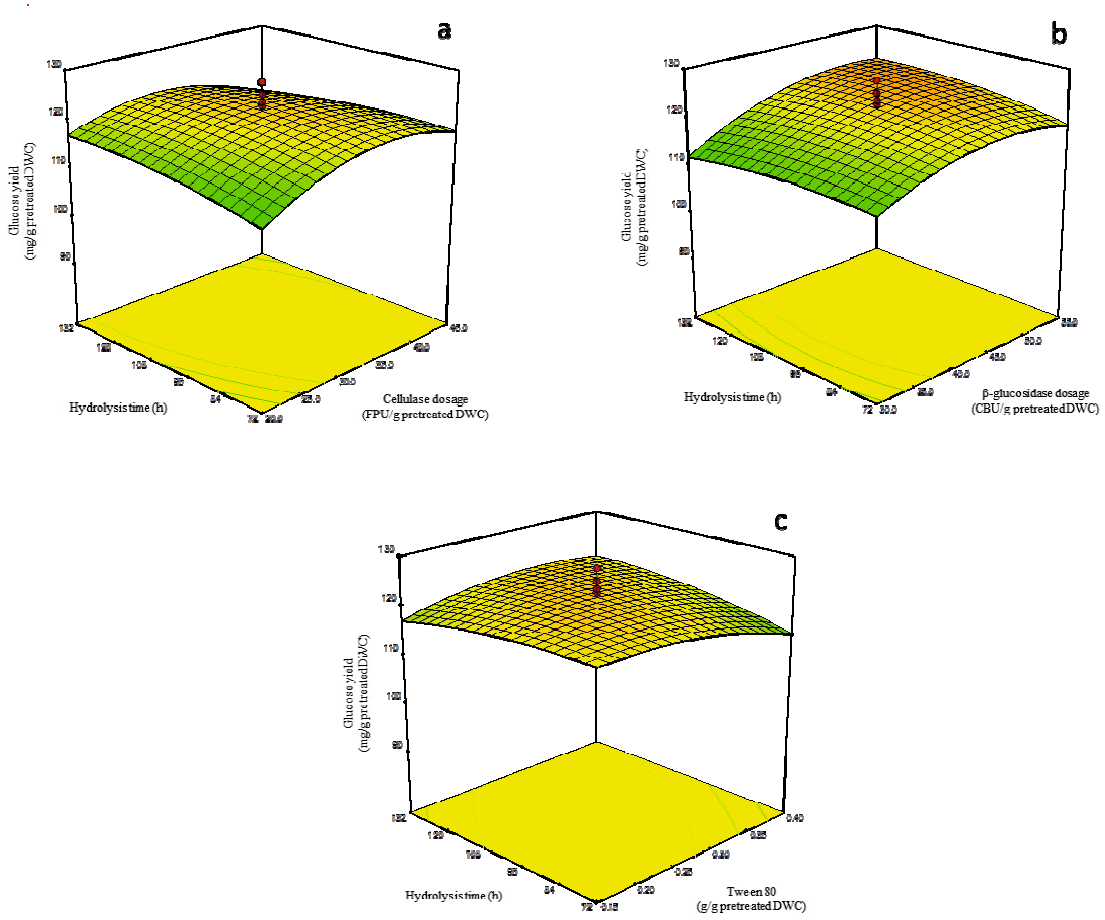

Fig. 4.1 - Response surface curves and contour plots of glucose yield from enzymatic hydrolysis: interaction between (a) cellulase dosage and Tween 80; (b) β-glucosidase dosage and hydrolysis time; (c) Tween 80 and hydrolysis time.

supplement of β-glucosidase, varied by hydrolysis time, could prevent cellobiose accumulation and enzyme inhibition in the system (Fang et al., 2010; Singh and Bishnoi, 2012a). The interaction effect of Tween 80 and hydrolysis time on glucose and reducing sugar yields is illustrated in **Figures 4.1c** and **4.2c**, respectively. Glucose was found to have a low yield at a high level of Tween 80 (0.4 g/g pretreated DWC) with a low level hydrolysis time (72 h) while reducing sugar was found to have a high yield. This phenomenon may explain that the hydrolysis process needs a longer time to hydrolyse the cellobiose to achieve the end-product glucose. Previous studies reported that the addition of surfactant to enzymatic hydrolysis of lignocellulose biomass, could improve the conversion of cellulose into soluble sugars because surfactant adsorbs onto lignin and prevents non-production enzymes binding on lignin (Kristensen et al., 2007). Moreover, Kaar and Holtzapple (1998) reported that surfactants could stabilize enzymes and prevent denaturation during hydrolysis.

Figure 4.2a shows the positive interaction effect between β-glucosidase dosage and Tween 80. Reducing sugar yield was found to be at maximum with a high level of β-glucosidase dosage and Tween 80. It seems possible that these results are due to the addition of surfactant, which may reduce the unproductive enzyme adsorption onto lignin and increase adsorption of cellulase on the biomass (Eriksson et al., 2002). In addition, the supplement of β-glucosidase may help to cleave cellobiose into glucose to enhance the enzymatic hydrolysis.

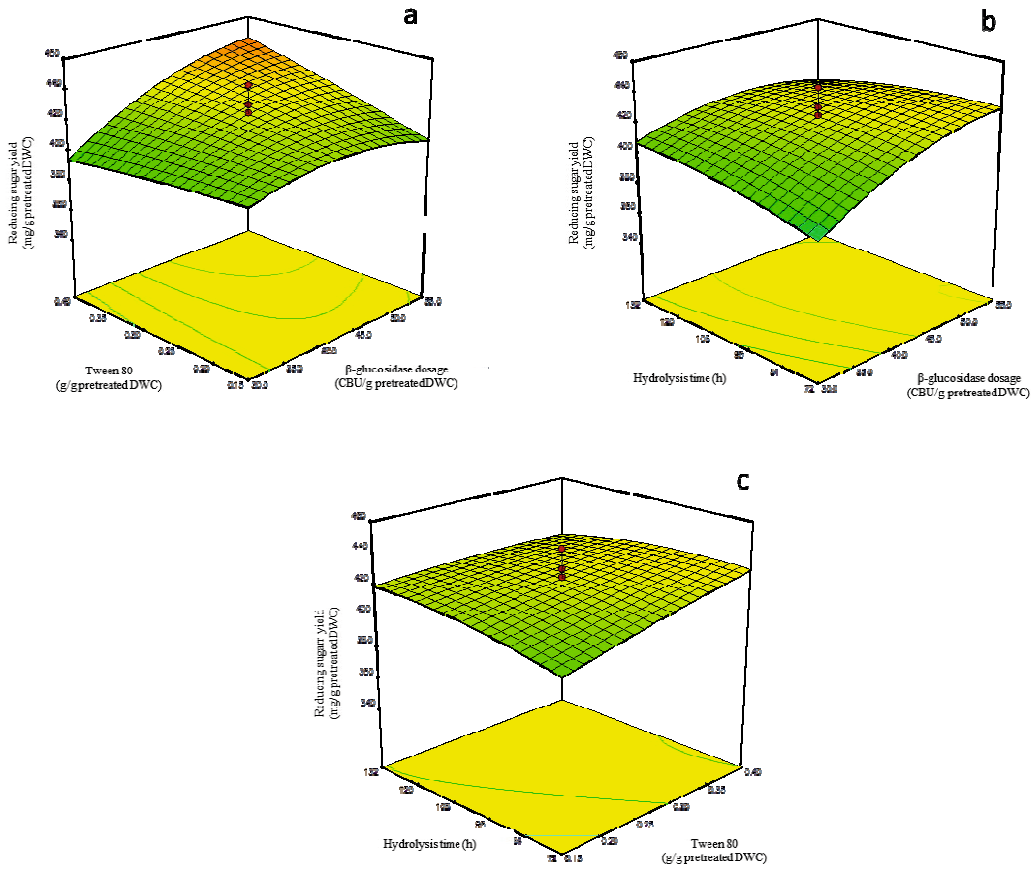

Fig. 4.2 - Response surface curves and contour plots of reducing sugar yield from enzymatic hydrolysis: interaction between (a) β-glucosidase dosage and Tween 80; (b) β-glucosidase dosage and hydrolysis time; (c) Tween 80 and hydrolysis time.

The optimal condition for enzymatic hydrolysis was predicted by Design Expert® as follows: 36 FPU/g pretreated DWC of cellulase dosage, 53 CBU/g pretreated DWC of β-glucosidase, 0.4 g/g pretreated DWC of Tween 80 for 105 h of hydrolysis time. The predicted glucose yield and reducing sugar yield calculation based on Eqs. 4.2 and 4.3 were 121.5 and 441.8 mg/g pretreated DWC and are in good agreement with the experimental responses of 121.7 and 435.8 mg/g pretreated DWC, respectively. It was confirmed that the models used were appropriately applicable to predict the experimental results. Theoretical

yields of glucose and reducing sugar, based on a 47% cellulose and 16% hemicellulose content in DWC were 520 and 706 mg/g DWC, respectively. Under optimal conditions, 24% and 62% of glucose and reducing sugar conversion yield were obtained in this study, respectively. **Table 4.5** shows a comparison between enzymatic hydrolysis in hardwoods and the hydrolysis of an olive tree pretreated by stream explosion, combined with alkaline pretreatment producing a yield of 288 and 363 mg/g biomass of reducing sugar, respectively (Cara et al., 2006, 2008). Celluclast 1.5L and Novozyme 188 were applied to dilute acid pretreated aspen corresponding to the reducing sugar yield of 507 mg/g biomass (Jensen et al., 2010). Surfactant, Polyethylene glycol (PEG) 6000, was introduced in the enzymatic hydrolysis of aspen and released 429 mg/g biomass (Jagtap et al., 2013). The results of this study show that the optimization technique could successfully improve enzymatic hydrolysis, according to the higher reducing sugar yield released in hydrolysate, when compared to most of the previous studies.

Table 4.5 - Comparison of enzymatic hydrolysis in hardwoods by various enzyme sources

Biomass	Pretreatment	Enzymatic hydrolysis condition	Reducing sugar yield (mg/g substrate)	References
Olive tree	Steam Explosion and Alkaline Peroxide Pretreatment	15 FPU/ g substrate Celluclast 1.5L; 12.6 IU/ g substrate Novozyme 188 for 72 h	288	Cara et al. (2006)
Olive tree	Dilute Acid Pretreatment	15 FPU/ g substrate Celluclast 1.5L; 15 IU/ g substrate Novozyme 188 for 72 h	363	Cara et al. (2008)
Aspen	Dilute Acid Pretreatment	32 FPU/g substrate Spezyme CP; 63 CBU/g substrate Novozyme 188 for 72 h	507	Jensen et al. (2010)
Aspen	Alkaline Pretreatment	35 FPU/ g substrate *Armillaria geminas* supplement with 0.75 mL/g substrate PEG 6000 for 48 h	429	Jagtap et al. (2013)
DWC	Alkaline Pretreatment	36 FPU/g substrate Celluclast 1.5L, 53 CBU/g substrate Novozyme 188, 0.4 g/g substrate Tween 80 for 105 h	436	This study

4.3.3 H_2 production from pretreated DWC hydrolysate

Enzymatic hydrolysate of pretreated DWC was applied as a substrate in fermentative hydrogen production from a hot spring mixed culture as shown in **Table 4.6**. The hydrolysate consisted of 6.1 g/L glucose, 3.5 g/L xylose and 21.8 g/L reducing sugar. A modified Gompertz equation was used to calculate cumulative H_2, maximum hydrogen production rate (HPR), and lag time, which were obtained 1,297 mL H_2/L, 23 mL H_2/L·h and 82 h respectively in the present study. Since the lag time is relatively long, it may suggest that surfactant exists in the enzymatic hydrolysate. Consequently, H_2-producing bacteria may need a longer time to adapt to the new environment; microbial acclimatization could shorten the lag time of H_2-producing bacteria. H_2 production yield (HY) was 27 mL H_2/g pretreated DWC (22 mL H_2/g DWC). Fermentative H_2 production was accompanied by the formation of the soluble metabolites, acetic acid, and butyric acid, were observed at 25.7 and 0.5 mM in this study, respectively.

Table 4.6 - Performance of fermentative H_2 production by the anaerobic mixed culture using enzymatic hydrolysate of pretreated DWC as a substrate.

Glucose (g/L)		Xylose (g/L)		Reducing Sugar (g/L)		Cumulative H_2 (mL H_2/L)	Maximum HPR (mL H_2/L·h)	Lag time (h)	R^2	HY (mL H_2/g pretreated DWC)	Soluble Metabolites (mM)	
Initial	Final	Initial	Final	Initial	Final						HAc	HBu
6.1	0	3.5	2.7	21.8	5.9	1297±14	23±0.7	82±3	0.9970	27±0.6	25.7	0.5

H_2 production yield from various lignocellulosic hydrolysates are summarized in **Table 4.7**. H_2 production yield from the acid hydrolysates were found lower than the enzymatic hydrolysate as compared in the literature. This is supported by the finding of Cui et al., (2010), who investigated the effect of different pretreatment method on H_2 production from poplar leaves, and found that H_2 yield from enzymatic hydrolysate (45 mL H_2/g dry biomass) was 1.3-fold higher than acid hydrolysate (34 mL H_2/g dry biomass). Lower H_2 yield from acid hydrolysate was possibly resulted by fermentation inhibitory compounds, furfural and hydroxyl methyl furfural, which were generated during acid hydrolysis (Xia et al., 2013). Thus, enzymatic hydrolysate could suitably serve as a substrate for fermentative H_2 production because of no or small amounts of inhibitory compounds and ease of operation. However, the H_2 production yield obtained in this study is lower than previous reports.

According to our knowledge, this study is the first report of H_2 production from enzymatic hydrolysate of DWC so far, hence more research on this topic needs to be undertaken such as optimization of culture medium in order to favor the efficiency of H_2-producing bacteria to enhance H_2 production.

Table 4.7 - H_2 production yield from various lignocellulosic hydrolysates

Substrate	Pretreatment	Culture	H_2 Yield	References
Rice straw	Concentrated acid hydrolysis	Municipal wastewater treatment sludge	0.44 mol H_2/mol sugar	Liu et al., (2013)
Rice straw	Diluted acid hydrolysis	Municipal sewage treatment sludge	0.305 mmol H_2/g rice straw	Chang et al., (2011)
Poplar leaves	Diluted acid hydrolysis	Mixed cultures from cracked cereals	33.45 mL H_2/g dry biomass	Cui et al., (2010)
Poplar leaves	Enzymatic hydrolysis	Mixed cultures from cracked cereals	44.92 mL H_2/g dry biomass	Cui et al., (2010)
Rice straw	Alkaline pretreatment followed by enzymatic hydrolysis	*Clostridium butyricum* CGS5	0.76 mol H_2/mol xylose	Lo et al., (2010)
Oat straw	Diluted acid and alkaline pretreatment followed by enzymatic hydrolysis	Anaerobic granular sludge	0.81 mol H_2/mol sugar	Arreola-Vargas et al., (2013)
DWC	Alkaline pretreatment followed by enzymatic hydrolysis	Anaerobic mixed culture from Hot spring	0.55 mol H_2/mol glucose-equivalent 21.6 mL H_2/g DWC	This study

4.3.4 Evaluation of operation cost of enzymatic hydrolysis condition

In order to develop the process to be more feasible in practice, there was not only recovery of reducing sugar yield needed to be considered but also cost of operation process. **Table 4.8** shows comparison operation cost in different condition based upon three scenarios. Each condition and sugar yields were predicted based on Eq. (4.2) and (4.3) using Design Expert®. Scenarios were set by the following criteria: Scenario I optimized the condition in all factors to get maximum reducing sugar yield (optimum condition); Scenario II minimized the dosages of both enzymes; Scenario III minimized the dosages of both enzymes and hydrolysis time. Glucose yield was reduced 3% in scenario II and III as compared with the

optimum condition. Reducing sugar yield was decreased 8% and 9% in scenario II and III, respectively, as compared with the optimum condition. Reduction of sugar yield was relatively similar in scenario I and II, whereas the reduction of total operation cost in scenario III (28%) was significantly lower than in scenario II (13%). Scenario III is apparent to be the most efficiency condition considering based on operation cost and performance, therefore the condition of scenario III was selected to be applied in the Chapter 5 later. From the results of this study, RSM could be used as a tool to estimate the operation performance within the experimental design ranges, in which, could be evaluated with the other aspect making the process more applicable in practice.

Table 4.8 - Operation cost of enzymatic hydrolysis in different scenarios

Factors	Unit	Condition		
		Optimum	Scenario II	Scenario III
Cellulase dosage	FPU/g pretreated DWC	36	26	28
	USD/kg pretreated DWC	6.0	4.3	4.7
B-glucosidase dosage	CBU/g pretreated DWC	53	37	38
	USD/kg pretreated DWC	8.0	5.6	5.7
Tween 80	g/g pretreated DWC	0.38	0.26	0.26
	USD/kg pretreated DWC	0.8	0.5	0.5
Hydrolysis time	h	105.0	116.0	72.0
	USD/kg pretreated DWC	10.4	11.5	7.1
Total cost	USD/kg pretreated DWC	25.1	21.9 (-13%)	18.0 (-28%)
Glucose Yield	g/ kg pretreated DWC	121.7	111.0 (-3%)	118.2 (-3%)
Reducing Sugars Yield	g/ kg pretreated DWC	435.8	407.0 (-8%)	403.2 (-9%)

4.4 Conclusions

The enzymatic hydrolysis of pretreated DWC was optimized by using RSM in this study. Reducing sugar yield was obtained at 435.8 mg/g pretreated DWC (348.6 mg/g DWC) under optimal conditions; 36 FPU/g pretreated DWC of cellulase dosage, 53 CBU/g pretreated DWC of β-glucosidase, 0.4 g/g pretreated DWC of Tween 80 for 105 h of hydrolysis time. H_2 yield was obtained at 27 mL H_2/g pretreated DWC (22 mL H_2/g DWC) from enzymatic hydrolysate. Based on the results of this present work, DCW possibly serves

as an alternative feed stock in fermentative H_2 production.

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CHAPTER V

STATISTICAL OPTIMIZATION OF MEDIUM COMPONENTS FOR HYDROGEN PRODUCTION FROM WOODEN HYDROLYSATE BY THERMOPHILIC ANAEROBIC MIXED CULTURE

5.1 Introduction

H_2 is considered as a promising clean energy carrier for the future. The main advantage of using H_2 as an energy carrier is that it is clean: fewer greenhouse gases were emitted during the production and combustion (Cheng et al., 2011). In addition, H_2 contained a high energy content (122 kJ/g), which is 2.75 times greater than hydrocarbon fuel (Hawkes et al., 2007). There are several H_2 production processes; however, H_2 production through dark fermentation has been an attractive option for decades. The significant advantage of dark fermentation is that various kinds of organic waste or lignocellulosic biomass can be utilized as a substrate for H_2 production. Lignocellulosic biomass is a recent trend as a promising renewable feed-stock for fermentative hydrogen production due to its cost-effectiveness and abundance in nature (Kim and Dale, 2004). Wooden chopsticks are one of the major sources of wood waste in East Asian countries such as Taiwan, China, and Japan. The annual average amount of waste produced by the disposable wooden chopsticks (DWC) was approximately 90,000 tons in Japan (Asada et al., 2011). Since a large amount of wooden waste is generated annually, there is the possibility that DWC can be used as a renewable low-cost feed stock for fermentative H_2 production in terms of conversion of waste to energy.

H_2 production via dark fermentation is influenced by many factors e.g., temperature, pH, and medium composition. There are several components in the culture medium that may significantly affect H_2 production. The nitrogen source is an essential component for proteins, nucleic acids, and enzyme synthesis (Wang and Wan, 2009), which is necessary for bacterial activity and growth. Srikanth and Venkata Mohan (2012) reported that magnesium plays an important role in the metabolic processes for substrate utilization. Magnesium is also a component of cell walls and cell membranes and a cofactor of many enzymes involved in the glycolysis pathway (Wang et al., 2007). Iron and Nickel are the main components on the active site of hydrogenase, which is the key enzyme in dark fermentation (Vignais and Billoud, 2007 and Hallenbeck and Ghosh, 2009). Trace elements are required for the

activation or function of several enzymes and coenzymes related to energy metabolism e.g., manganese (Srikanth and Venkata Mohan, 2012), zinc (Zheng and Yu, 2004), copper (Zheng and Yu, 2004), and calcium (Yuan et al., 2010). In the anaerobic process, a reducing agent like L-cysteine is required to reduce the oxidation-reduction potential in the culture medium (Song and Logan, 2004). Yuan et al., 2008 and Bao et al., 2013 investigated whether the addition of L-cysteine could enhance H_2 productivity. During the dark fermentation, volatile fatty acids (VFAs) were also produced to accompany H_2; the pH in the culture medium was dropped and caused an adverse effect on cell growth and H_2 production. A buffer needs to be added to the culture medium in order to avoid the rapid decrease of pH in the process. A phosphate buffer not only has the buffer capacity but is also used as a phosphate source for microbial growth (Lin and Lay, 2004). Since there are several medium components that might influence the H_2 production, medium composition levels are needed in order to maximize the H_2 production. Response surface methodology (RSM) was used to design the experiment and determine the optimal conditions for the desired responses. This method has many advantages over the conventional method (one factor at a time) in terms of time saving and a reduction in the work required, together with the opportunity to examine the interaction amongst variables (Bezerra et al., 2008).

The objective of this study was to screen the significant medium components affecting H_2 production using a Plackett-Burman design (PBD). Subsequently, the levels of the key components were optimized by a central composite design (CCD) in order to enhance H_2 production from wooden hydrolysate by a thermophilic anaerobic mixed culture.

5.2 Materials and methods

5.2.1 Raw material

DWC waste was collected from the cafeteria at the Faculty of Science and Engineering, Yamaguchi University, Yamaguchi Prefecture, Japan. DWC were soaked in tap water overnight and container. washed with detergent, then dried at 45°C overnight. Dried DWC were ground in a mill (LM-Plus, Osaka Chemical, Osaka, Japan) and passed through a 2 mm screen, then stored at room temperature in a sealed

5.2.2 Alkaline pretreatment and enzymatic hydrolysis

10% (w/v dry mass) of DWC was pretreated with 2% NaOH at 100 °C for 30 minutes (Phummala et al., 2014). Subsequently, pretreated DWC (cellulose fractions) was hydrolyzed

by enzyme. The conditions for enzymatic hydrolysis in this present work involved 28 FPU/g pretreated DWC of cellulase, 38 CBU/g pretreated DWC of β-glucosidase, and 0.26 g/g pretreated DWC of Tween 80 for 72 h. The mixture was incubated at 50 °C. At the end of incubation, samples were immediately heated to 100 °C to denature the enzymes, cooled, and then centrifuged for 10 minutes at 7,380×g. Reducing sugar concentration in hydrolysate involved 20.1 g/L, which consisted of 5.8 g/L glucose, 3.5 g/L xylose, and 10.5 g/L unknown reducing sugar. The hydrolysate was used as a substrate for fermentative H_2 production.

5.2.3 Inoculum and H_2 fermentation

Anaerobic mixed culture was enriched from a hot spring in Beppu, Oita Prefecture, Japan. Inoculum was prepared under anaerobic conditions in a medium containing 5 g/L each of glucose and xylose, 1 g/L of yeast extract, 0.5 g/L of L-cysteine, 0.3 g/L $MgCl_2.6H_2O$, 0.25 g/L KH_2PO_4, 0.25 g/L K_2HPO_4, 0.025 g/L $FeSO_4.7H_2O$, 0.016 g/L $NiCl_2.6H_2O$, and 1 mL/L of trace elements containing 25 $CoCl_2.6H_2O$, 15 $MnCl_2.4H_2O$, 11.5 $ZnCl_2$, 10.5 $CuCl_2$, and 5 $CaCl_2$ in g/L. The pH of medium was adjusted to 7.0 and incubated at 50 °C for 24 hours. The experiment was conducted in 30 mL serum bottles with a working volume of 15 mL. Thirty percent of inoculum was centrifuged at 2,860×g for 15 minutes before inoculation into the hydrolysate supplement with the desired concentration of medium components based on the experimental design. The serum bottles were fitted with rubber stoppers and aluminum caps, and then flushed with N_2 gas to obtain anaerobic conditions. The experiment was conducted in duplicate at the optimal pH and temperature, as mentioned above. Biogas was monitored periodically until none was produced.

5.2.4 Experimental design and optimization

5.2.4.1 Plackett-Burman design

PBD was applied for screening the important factors that significantly impacted on hydrogen production. The design was based on first order model:

$$Y = \beta_0 + \sum \beta_i x_i \quad (5.1)$$

where Y is the response (hydrogen production potential, P), β_0 is the model intercept, β_i is the linear coefficient and x_i is the level of the independent variables. Seven medium components were investigated as the key ingredients for hydrogen production. The level of each

component was set at two levels: -1 for low level and +1 for high level, and the experimental design including the response is presented in **Table 5.1**.

Table 5.1 - Plackett-Burman design of variables (in coded levels) with potential H_2 production (P) as response

Run	Variables							Responses
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	P (mL H_2/L)
1	1	-1	1	-1	-1	-1	1	856±29
2	1	1	-1	1	-1	-1	-1	1599±14
3	-1	1	1	-1	1	-1	-1	921±5
4	1	-1	1	1	-1	1	-1	1087±136
5	1	1	-1	1	1	-1	1	1615±26
6	1	1	1	-1	1	1	-1	1205±56
7	-1	1	1	1	-1	1	1	903±28
8	-1	-1	1	1	1	-1	1	543±49
9	-1	-1	-1	1	1	1	-1	454±77
10	1	-1	-1	-1	1	1	1	834±33
11	-1	1	-1	-1	-1	1	1	922±43
12	-1	-1	-1	-1	-1	-1	-1	499±27

5.4.2.2 Path of steepest ascent

The path of steepest ascent was performed in order to approach the area of the optimum, the direction of steepest ascent is the direction in which the response was increased or decreased by increasing of significant factors. The zero level of PBD was initially identified as the base point of steepest ascent path. The experiments were conducted along the steepest ascent path until the response had no longer increase. The maximum response was assumed to be the vicinity of the optimal point and used as the central point for optimization in the further step.

5.2.4.3 Central composite design

Based on the PBD results, three components, yeast extract (X_1), phosphate buffer (X_2), and $FeSO_4 \cdot 7H_2O$ (X_4) were identified as significantly influencing hydrogen production. Hence, the three key components were selected to be subsequently investigated for the optimal levels by CCD. In this study, each component was assessed at five different levels, including two factorial points (-1, 1), two axial points (-1.68, 1.68), and a central point (0). A total of 20 experiments were conducted as shown in **Table 5.2**. The relationship between the coded values and actual values are described as follows:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \tag{5.2}$$

where x_i is the coded value of an independent variable X_i, X_i is the actual value of an independent variable, X_0 is the actual value of an independent variable at the center point, and ΔX_i is the step change of variables. The optimal concentration of three key components was predicted based on the secondary order model:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j \tag{5.3}$$

where Y is the predicted response; β_0 is the interception coefficient; β_i is the linear coefficient, β_{ii} is the quadratic coefficient; β_{ij} is the interactive coefficient; and X_i and X_j are the independent variables. The Design Expert 9.0.3.1 (Trial Version, Stat-Ease Inc., Minneapolis, USA) software was used for experimental design, regression, and graphical analysis of the experimental data.

5.2.5 Analytical methods

Hydrogen production was measured by periodically by using a wet glass syringe. Hydrogen content in biogas was analyzed using a GC-8APT gas chromatograph, equipped with a thermal conductivity detector (TCD) (Shimadzu, Kyoto, Japan) (Phummala et al., 2014). The cumulative H_2 production data of each test was fitted to a modified Gompertz equation (Khanal et al., 2004). At the end of incubation, residual glucose and xylose were determined by by HPLC (Phummala et al., 2014) and residual reducing sugar was analyzed by the dinitrosalicylic acid (DNS) method. Metabolites soluble, volatile fatty acids (VFAs), were determined by a GC-8APF gas chromatograph equipped flame ionization detector (FID) (Shimadzu, Kyoto, Japan) (Phummala et al., 2014).

Table 5.2 - Experimental design and results of CCD

Run	Yeast extract (X_1)		Phosphate Buffer (X_2)		$FeSO_4 \cdot 7H_2O$ (X_4)		P (mL H_2/L)	
	Coded	Actual (g/L)	Coded	Actual (mM)	Coded	Actual (g/L)	Experiment	Predicted
1	-1	2	-1	25	-1	0.050	1140±33	1118
2	1	7	-1	25	-1	0.050	2039±45	1993
3	-1	2	1	75	-1	0.050	934±41	870
4	1	7	1	75	-1	0.050	1142±110	1203
5	-1	2	-1	25	1	0.100	951±11	893
6	1	7	-1	25	1	0.100	1711±0.2	1779
7	-1	2	1	75	1	0.100	808±112	857
8	1	7	1	75	1	0.100	1174±6	1200
9	-1.68	0.3	0	50	0	0.075	528±16	586
10	1.68	8.7	0	50	0	0.075	1674±12	1611
11	0	4.5	-1.68	7.96	0	0.075	1402±91	1439
12	0	4.5	1.68	92.04	0	0.075	786±0.3	745
13	0	4.5	0	50	-1.68	0.033	1665±32	1710
14	0	4.5	0	50	1.68	0.117	1568±1	1519
15	0	4.5	0	50	0	0.075	1744±8	1752
16	0	4.5	0	50	0	0.075	1702±86	1752
17	0	4.5	0	50	0	0.075	1810±1	1752
18	0	4.5	0	50	0	0.075	1809±12	1752
19	0	4.5	0	50	0	0.075	1670±20	1752
20	0	4.5	0	50	0	0.075	1775±16	1752

5.3 Results and discussions

5.3.1 Screening significant medium components affecting H_2 production by PBD

A total of seven medium components were investigated for their effect on hydrogen production and the significant medium components were screened using PBD. The experimental design and corresponding responses are shown in **Table 5.1**. **Table 5.3** illustrates the effect of each medium component on the potential hydrogen production (P), yeast extract (X_1), phosphate buffer (X_2), and $FeSO_4 \cdot 7H_2O$ (X_4) showed a positive effect indicating that increasing the level of those variables from low to high increased the potential hydrogen production, whereas $MgCl_2 \cdot 6H_2O$ (X_3), $NiCl_2 \cdot 6H_2O$ (X_5), trace elements (X_6), and L-cysteine (X_7) revealed negative effect on the potential hydrogen production. Analysis of variance (ANOVA) was employed in which the medium component had a confidence level above 95% to be considered as a significant medium component for hydrogen production.

Yeast extract (X_1), phosphate buffer (X_2), and $FeSO_4 \cdot 7H_2O$ (X_4) were examined as significant medium components. Only these three key components were subsequently determined for the optimal levels in the further step, while the other four components were found to be insignificant and neglected in the optimization.

Table 5.3 - Levels of variables and statistical analysis of PBD

Factors	Low level (-1)	High level (+1)	Effect (E_{xi})	Coefficient	*F*-value	*p*-value
Model				953.14	27.77	0.0031*
Yeast extract (g/L)	1	3	492.50	246.25	90.92	0.0007*
Phosphate buffer (mM)	1	50	481.72	240.86	86.98	0.0007*
$MgCl_2 \cdot 6H_20$ (g/L)	0.3	0.5	-68.06	-34.03	1.74	0.2580
$FeSO_4 \cdot 7H_2O$ (g/L)	0.025	0.075	160.95	80.47	9.71	0.0357*
$NiCl_2 \cdot 6H_2O$ (g/L)	0.016	0.032	-48.94	-24.47	0.9	0.3970
Trace element (mL/L)	1	3	-104.39	-52.20	4.08	0.1134
L-cysteine (g/L)	0.5	1	-15.22	-7.61	0.087	0.7829

$R^2 = 0.9798$; Adjusted-$R^2 = 0.9446$

* 95% confidence level.

5.3.2 Path of steepest ascent

Based on the results of PBD, the path of steepest ascent was performed to approach the vicinity of optimal area, the concentration of yeast extract, phosphate buffer and $FeSO_4 \cdot 7H_2O$ were increased in order to enhance hydrogen production. In **Table 5.4**, the highest P was reached approximately 1,704 – 1,713 mL H_2/L with the range concentration of yeast extract 4-5 g/L; phosphate buffer 45-55 mM and $FeSO_4 \cdot 7H_2O$ 0.07-0.08 g/L. Thus, this area might be the area of optimal point and was further optimized in the next step.

Table 5.4 - Experimental design and results of path of steepest ascent

Run	Yeast extract (g/L): X_1	Phosphate buffer (mM): X_2	$FeSO_4 \cdot 7H_2O$ (g/L): X_3	P (mL H_2/L)
1	2	25	0.05	1049±17
2	3	35	0.06	1427±29
3	4	45	0.07	1704±49
4	5	55	0.08	1713±6
5	6	65	0.09	1391±11
6	7	75	0.1	962±32

5.3.3 Optimization of medium composition for H_2 production

The concentrations of yeast extract, phosphate buffer and $FeSO_4 \cdot 7H_2O$ were optimized by CCD in order to maximize the hydrogen production, and determine the potential interactive effect of these three significant components. The insignificant components ($MgCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, trace elements and L-cysteine) were kept at low levels in accordance with their negative effects on H_2 production. The experimental design of CCD and corresponding experimental results was tabulated in **Table 5.2**. According to regression analysis, the experimental results fitted adequately with the secondary order model as shown in the following equation:

$$Y = 506.03X_1 + 32.49X_2 + 4986.59X_4 - 36.97X_1^2 - 0.37X_2^2 - 77848.60X_4^2 - 2.17X_1X_2 + 40.72X_1X_4 + 84.68X_2X_4 - 489.69 \quad (5.4)$$

where, Y is potential H_2 production, X_1, X_2 and X_4 are the actual values of yeast extract, phosphate buffer, and $FeSO_4 \cdot 7H_2O$, respectively.

The predicted values of P were calculated based on Eq. (5.4) and given in **Table 5.2** along with the experimental results. ANOVA was used to evaluate the significance of the fit of the secondary order model for hydrogen production as shown in **Table 5.5**. *F*-value of the model was 73.48 and *p*-value was less than 0.0001 implied that the model was significant ($p<0.05$). In contradiction, *F*-value for lack of fit was 2.23 and *p*-value was 0.1995 indicating that the lack of fit was insignificant ($p>0.05$). Thus, the obtained experiment data was a good

fit with the model. The coefficient of determination (R^2=0.9851) explained that the model is capable, attributing 98.51% to the variability. The predicted determination coefficient (Pred R^2=0.9103) is in reasonable agreement with the adjusted determination coefficient (Adj R^2=0.9717), which is also satisfactory to confirm the fitness of the model. Based on ANOVA results, all three components showed both linear and quadratic effects on H_2 production. Yeast extract had an interactive effect with a phosphate buffer, whereas $FeSO_4 \cdot 7H_2O$ had an insignificant interactive effect on the other two components.

Table 5.5 - ANOVA for the model regression of CCD

Source	Sum of Squares	DF	Mean Square	*F*-Value	*p*-value
Model	3.48E+06	9	3.87E+05	73.48	< 0.0001*
X_1	1.27E+06	1	1.27E+06	240.95	< 0.0001*
X_2	5.83E+05	1	5.83E+05	110.81	< 0.0001*
X_4	44118.12	1	44118.12	8.39	0.0159*
X_1^2	7.69E+05	1	7.69E+05	146.25	< 0.0001*
X_2^2	7.85E+05	1	7.85E+05	149.19	< 0.0001*
X_4^2	3.41E+04	1	3.41E+04	6.49	0.029*
X_1X_2	1.47E+05	1	1.47E+05	27.99	0.0004*
X_1X_4	5.18E+01	1	5.18E+01	9.85E-03	0.9229
X_2X_4	22408.32	1	22408.32	4.26	0.0659
Residual	52599.84	10	5259.98		
Lack of Fit	36318.27	5	7263.65	2.23	0.1996
Pure Error	1.63E+04	5	3.26E+03		
Cor Total	3.53E+06	19			

R^2 = 0.9851; Adjusted-R^2 = 0.9717; Predicted-R^2 = 0.9103

* 95% confidence level.

The response surface plots and contour plots are illustrated in **Fig. 5.1**. Two variables were varied within the design range, while the rest of the variables were kept constant at the center point. The H_2 production was increased by increasing the concentrations of yeast extract and phosphate buffer until the optimum levels were achieved and decreased thereafter. However, there is a slight difference on H_2 production while the concentration of $FeSO_4 \cdot 7H_2O$ changed. The results may suggest that an increased concentration of yeast extract and phosphate buffer was more effective than changing the concentration of $FeSO_4 \cdot 7H_2O$.

In order to achieve the optimum conditions, the maximum H_2 production was predicted at 2001 mL H_2/L using 6.74 g/L yeast extract, 29.62 mM phosphate buffer, and

0.05 g/L $FeSO_4 \cdot 7H_2O$. The experiment was conducted under optimal conditions in order to confirm the validity of the model. The experimental result of H_2 production was demonstrated at 1,949 mL H_2/L, which showed a good correlation with the predicted values (2001 mL H_2/L). Therefore, the results confirmed the validity of the model.

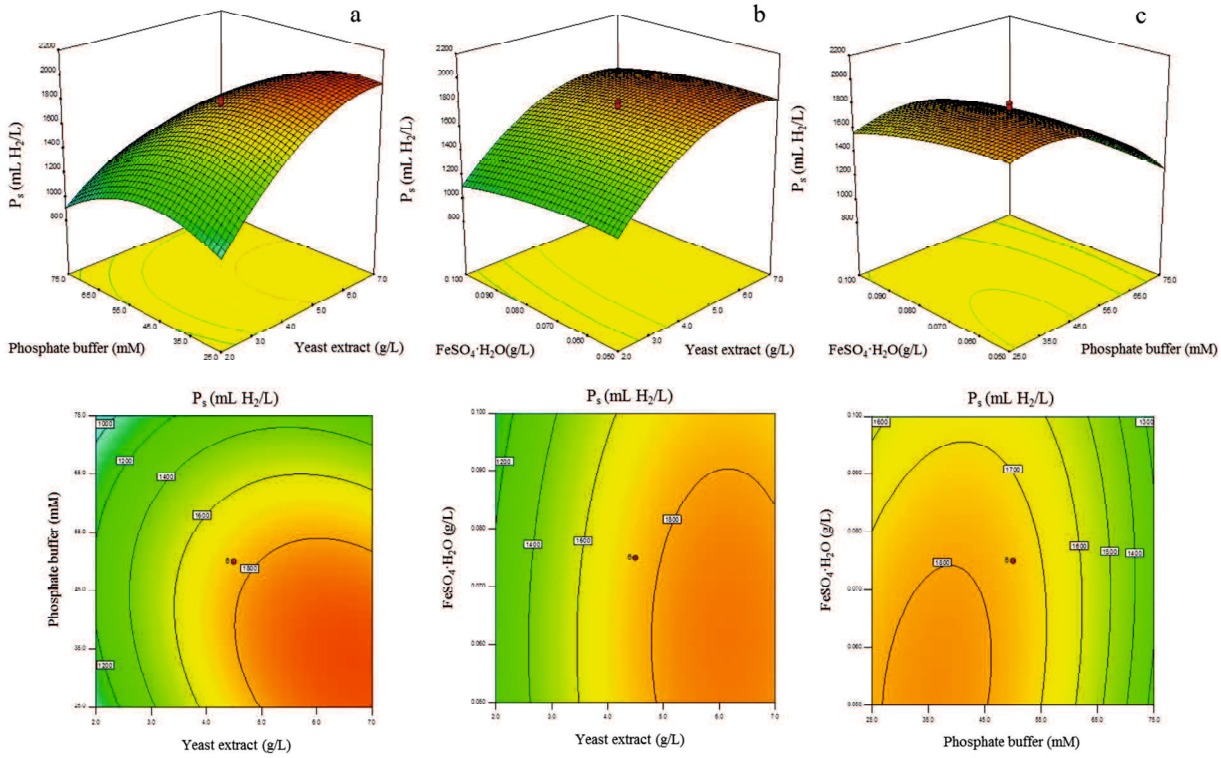

Fig. 5.1 - Three-dimensional response surface plots and two-dimensional contour plots of H_2 production illustrating interactions between: (a) yeast extract and phosphate buffer; (b) yeast extract and $FeSO_4 \cdot 7H_2O$; (c) phosphate buffer and $FeSO_4 \cdot 7H_2O$

Table 5.6 - Validation of the model

Run	Yeast extract (g/L)	Phosphate buffer (mM)	$FeSO_4$ $7H_2O$ (g/L)	P (mL H_2/L)		Maximum HPR (mL/L.h)	% Substrate consumed	Final pH	Soluble Metabolites (mM)		
				Experiment	Predicted				HAc	HBu	EtOH
Optimum	6.7	29.6	0.050	1954	2001	58	71.8	5.13	34.9	0.21	42.76
High	7	75	0.100	1071	1200	44	96.9	6.48	16.6	0.15	72.19
Medium	4.5	50	0.075	1727	1752	39	90.1	5.47	28.7	0.17	57.88
Low	2	25	0.050	1083	1117	32	46.0	4.84	12.3	0.00	0.00

Moreover, the high, medium and low levels of the medium components were also predicted in order to confirm the validity of the model (**Table 5.6**). The experiment under all condition were demonstrated and found that results of H_2 production were a good correlation with the predicted values.

Soluble metabolites were analyzed after the end of fermentation, results found that when yeast extract and phosphate buffer were varied at low level, only acetic acid was produced along with H_2 indicating that acetate type fermentation was dominant in the process (Eq. 5.5).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (5.5)$$

In contradiction, ethanol was generated increasingly accompany with H_2 when the concentration of yeast extract was increased together with increasing the concentration of phosphate buffer. So the fermentation pathway was possibly shifted from acetate to acetate-ethanol type fermentation that reduced the yield of H_2 production as shown in Eq. (5.6).

$$C_6H_{12}O_6 + H_2O \rightarrow CH_3COOH + CH_3CH_2OH + 2CO_2 + 2H_2 \quad (5.6)$$

5.4 Cost analysis of the overall process

In **table 5.7** shows the cost of operation of the overall process from the Chapter 3 – 5, the cost of chemical and energy consumption were basically calculated in each unit operation, alkaline pretreatment, enzymatic hydrolysis and H_2 production. The major contributor of chemical consumption cost was 36% of total taken in step of enzymatic hydrolysis. While the

energy cost was mainly distributed both on enzymatic hydrolysis and H_2 production processes which were taken approximately 62% in total. Overall cost of the operation process was 24.5 USD/kg DWC. **Table 5.8** shows a comparative study of cost analysis for various H_2 production by dark fermentation. Even though, the cost operation was considered and found relatively high in this study since the initial phase of development of H_2 production process from wood material was just investigated by this work. However, there is a feasibility to improve the fermentative H_2 production process by several aspects and efforts in order to be more applicable in the future.

Table 5.7 - Overall cost operation process

Unit Operation	Alkaline pretreatment	Enzymatic hydrolysis	H_2 production
Chemical Comsumption (USD/kg DWC)	0.10	8.73	0.43
Energy Consumption (USD/kg DWC)	0.07	5.69	9.48
Sub-total (USD/kg DWC)	0.17	14.42	9.91
		Total (USD/kg DWC)	24.51

Table 5.8 - Comparative study and cost analysis of various H_2 production by dark fermentation

Substrate	**Pretreatment**	**Production cost (USD/m^3)**	**Reference**
Sugarcane distillery effluent	na	35.4	Li et al., 2012
Beverage wastewater	na	7,100	Li et al., 2012
Agriculture waste	Two-step acid hydrolysis	7,900	Li et al., 2012
Solid bio-waste	Hydrolysis	1.52	Chang and Hsu, 2012
DWC	Alkali and enzymatic hydrolysis	766	This study

5.5 Conclusions

This study was an attempt to optimize the medium compositions for maximizing H_2 production from DWC hydrolysate. Seven medium components were screened using PBD, three out of seven components (yeast extract, phosphate buffer, and $FeSO_4 \cdot 7H_2O$) were found to have a significant effect on H_2 production. These components subsequently optimized their concentration via RSM using CCD. The maximum H_2 production was achieved at 1,949 mL H_2/L g pretreated DWC (1559 mL H_2/g DWC) under optimum conditions using 6.74 g/L yeast extract, 29.62 mM phosphate buffer and 0.05 g/L $FeSO_4 \cdot 7H_2O$. The results suggested that RSM is an applicable optimizing methodology for the H_2 production.

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CHAPTER VI
CONCLUSIONS AND FUTURE WORKS

6.1 Overall operation process performance and cost analysis

An overall operation process of fermentative H_2 production from wood material (DWC) was calculated based upon the experiment data obtained from the Chapter 3 – 5 and shows in **Fig. 6.1**. One kilogram of DWC at solid loading of 10% (w/v) was pretreated by 2% NaOH at 100°C for 30 min. Then, the solid fraction (cellulose fraction) was separated from the pretreated liquid fraction. The amount of recovered of material corresponded to approximately 80% (w/w) of the starting DWC and was feed for enzymatic hydrolysis. Enzymatic sacharification was performed under selected condition obtained from Chapter 4 with comprising of 26 FPU/g pretreated DWC cellulase, 38 CBU/g pretreated DWC β-glucosidase, 0.26 g/g pretreated DWC Tween 80 at 50°C for 72 h. Reducing sugar was yielded at 322 g/kg DWC (45% of theoretical sugar yield) in enzymatic hydrolysate which was then used as substrate for H_2 production by anaerobic mixed culture. H_2 production yield was 32 L H_2/kg DWC obtained under optimum medium and condition, soluble metabolites, acetic, butyric acids and ethanol, were produced along with H_2 at 33, 0.3, 32 g/kg DWC, respectively. Energy output was calculated based on the H_2 production yield and H_2 heating values. The total amount of energy was obtained approximately 313 kJ/kg DWC (34 % of potential theoretical energy output) in this study. Moreover, this process could be applied on other hardwood wastes i.e. wood chips, bark form forest residue; saw dust from furniture factory and wooden waste from demolished construction, etc. Even though, the cost operation was considered and found relatively high in this study since the initial phase of development of H_2 production process from wood material was just investigated by this work. However, there is a feasibility to improve the fermentative H_2 production process by several aspects and efforts in order to be more applicable in the future.

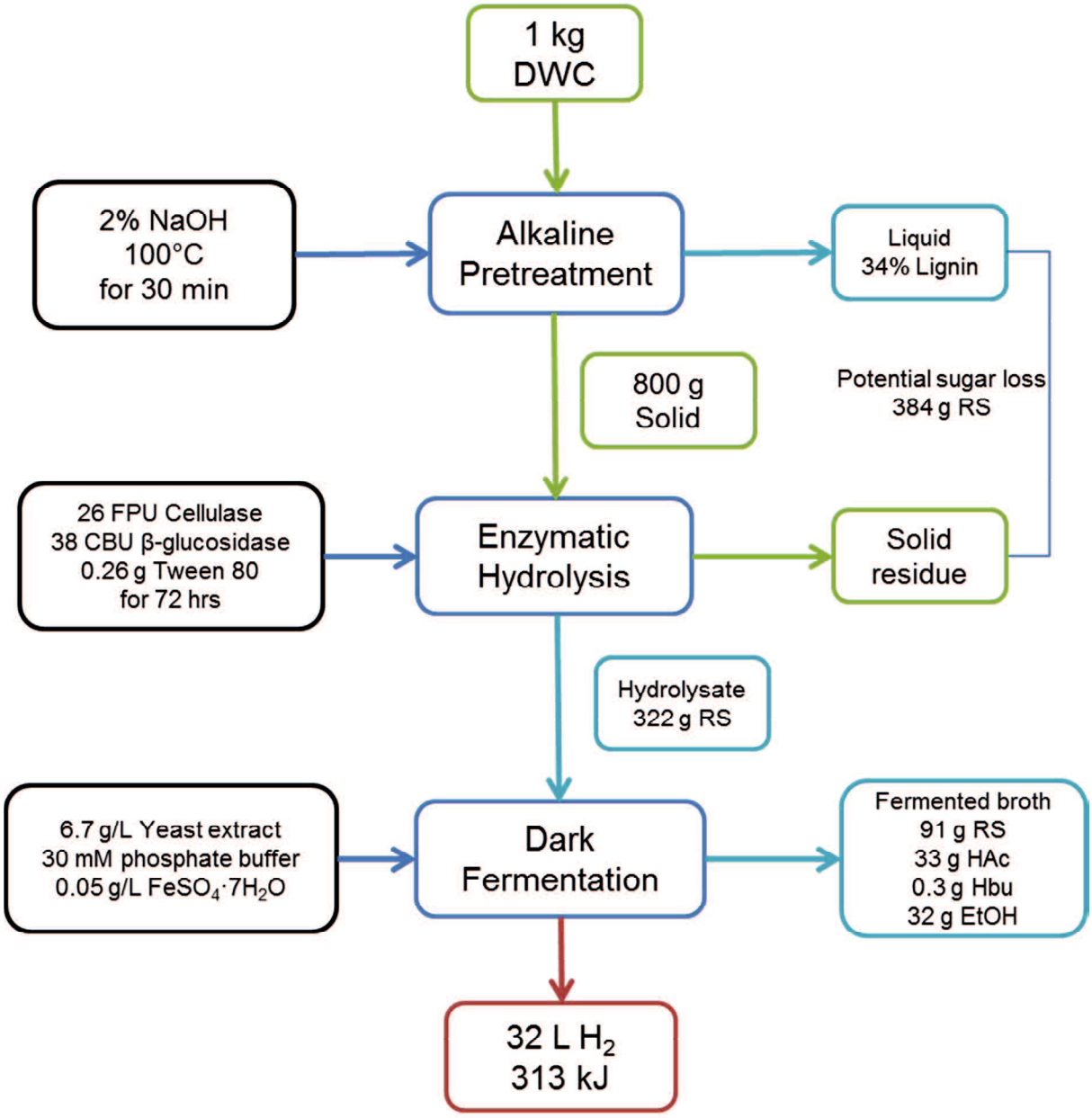

Fig.6.1 - Overall operational process performance of fermentative H_2 production from wood material (DWC)

6.2 Future works

Based on the results of this study, there is possibility of using DWC as a renewable feedstock for bio-H_2 production, however, H_2 production yield could be more improved in several efforts as listed below:

- Recovery of the enzyme should be employed in order to raise the feasibility of the enzymatic hydrolysis in practice. Immobilization technic may help this issue, this

technic makes enzyme easily in separation from the hydrolysate and possibly re-use in the process.

- According to the results of this study, DWC could not be completely converted into reducing sugars, about 54% theoretical yield of reducing sugars was still left in the residues and lost in the system. The residues are possibly further hydrolyzed to gain more reducing sugar yield.
- pH in culture medium was sharply dropped in batch fermentation of H_2 because of production of organic acids along with H_2 production resulting in H_2 has no longer produced in the system. To maintain the production of H_2, pH in the culture medium should be controlled during the fermentation.
- Dark fermentation is not a mineralization process of hydrolysate, soluble metabolites (such as organic acids and alcohol) are produced accompanying with H_2 as end products. In order to enhance efficiency of the process, two-stage fermentation which dark fermentation integrated with photo fermentation or methane fermentation in sequence would be more efficient not only for completely treating the end products but also improving the overall energy output yield.
- Due to the cost of energy consumption in enzymatic hydrolysis and H_2 fermentation was relatively high; this was equal to 62% of total cost of the process. One of the possible ways to reduce the energy consumption cost is applied simultaneous saccharification and fermentation (SSF) method substitute of separate hydrolysis and fermentation (SHF) method.
- Since lactic acid bacteria (LAB) was found in anaerobic mixed culture obtained from hot spring, LAB is a substrate competitor of H_2-producing bacteria. Therefore isolation of H_2-producing bacteria should be conducted in order to obtain a pure culture. H_2 fermentation by pure culture might be improved the H_2 production yield rather than mixed culture.
- Techno-economic feasibility should be further evaluated in more detail including fixed capital, operation, utility, labor and maintenance costs, revenue from H_2 sales and return period of investment.

APPENDIX

LIST OF PUBLICATION

1. **Phummala K.**, Imai T., Reungsang A., Higuchi T.,Sekine M., Yamamoto K., Kanno A. Optimization of enzymatic hydrolysis for pretreated wood waste by response surface methodology in fermentative hydrogen production. *Journal of Water and Environment Technology.* (Accept on October 15th, 2014)
2. Vo H. T., Imai T., Teeka J., Le T. V., **Phummala K.**, Higuchi T., Kanno A., Sekine M., Yamamoto K. **2014**. Potential application of pressurized carbon dioxide for agricultural irrigation water disinfection. *KKU Research Journal*, Vol. 19, xx-xx. (*In Press*)
3. **Phummala K.**, Imai T., Reungsang A., Chairattanamanokorn P., Sekine M., Higuchi T., Yamamoto K., Kanno A. **2014**. Delignification of disposable wooden chopsticks waste for fermentative hydrogen production by an enriched culture from a hot spring. *Journal of Environmental Sciences*, **26**(6), 1361–1368.
4. Vo H. T., Imai T., Teeka J., Le T. V., **Phummala K.**, Higuchi T., Kanno A., Sekine M. 2013. Comparison of disinfection effect of pressurized gases of CO_2, N_2O, and N_2 on *Escherichia coli*. *Water Research*, **47**(13), 4286-4293.

LIST OF PRESENTATION

1. **Phummala K.**, Imai T., Imai T., Higuchi T., Kanno A., Yamamoto K., Sekine M., Reungsang A. Statistical optimization of medium components for hydrogen production from wooden hydrolysate by thermophilic anaerobic mixed culture. *The 11th International Symposium on Southeast Asian Water Environment (SEAWE11).* Bangkok, Thailand. November 26th-28th, 2014. (Oral presentation)
2. **Phummala K.**, Imai T., Reungsang A., Higuchi T.,Sekine M., Yamamoto K., Kanno A. Optimization of enzymatic hydrolysis for pretreated wood waste by response surface methodology in fermentative hydrogen production. *Water and Environment Technology conference 2014*. Tokyo, Japan. June 28th-29th, 2014. (Oral and poster presentation)
3. **Phummala K.**, Imai T., Reungsang A., Chairattanamanokorn P. Delignification of disposable wooden chopsticks waste for fermentative hydrogen production by enriched culture from hot spring. *The 5th IWA- Aspire conference***.** Daejeon, Korea. September 8th – 12th, 2013. (Oral presentation).

4. **Phummala K**., Imai T., Reungsang A., Chairattanamanokorn P. Development of thermophilic hydrogen producing microbial consortia from hot springs using mixed of hexose and pentose sugars as the substrates. *Water and Environment Technology conference 2013*. Tokyo, Japan. June 15th -16th, 2013. (Oral and poster presentation).
5. Vo H. T., Imai T., Teeka J., Le T. V., **Phummala K.**, Higuchi T., Kanno A., Sekine M. Comparison of pressurized gas bubbles CO_2, N_2O and N_2 on *Escherichia coli* disinfection. *The 4th IWA young water professional Conference (APYWP2012).* Miraikan, Tokyo, Japan. December 7th-10th, 2012. (Poster presentation)
6. Vo H. T., Imai T., Teeka J., Le T. V., **Phummala K.**, Higuchi T., Kanno A., Sekine M.. Comparison of bactericidal effect on *Escherichia coli* by pressurized gases of CO_2, N_2O, and N_2. *The 6th young scientist seminar in Asian Core program.* Yamaguchi, Japan. September 9th-10th, 2012. (Oral presentation)