

CHAPTER I

INTRODUCTION

1.1 Rationale and Background

Renewable energy has received considerable attention because of a high price of the fossil fuels and the emission of greenhouse gases (CO and CO₂) from a utilization of fossil fuel (Kapdan and Kargi, 2006). Hydrogen is recognized as the most promising alternative to fossil fuels because of its high energy density of 122 kJ/g (2.75 times greater than the hydrocarbon fuel) (Han SK, Shin, 2004) and its environmentally friendly characteristic i.e. after its combustion with oxygen, water is the only by-product. Dark fermentative hydrogen production can utilize various kinds of renewable materials (e.g. agricultural waste, food waste, manufacturing wastewater of bio-industrial) as the substrates (Kelly-Yong et al., 2007; Hniman et al., 2011; Sreethawong et al., 2010; Sreela-or et al., 2011) with high hydrogen production rate and low cost of operation in comparison to photo-fermentation (Xie et al., 2010). Recently, a dark fermentative hydrogen production under thermophilic conditions has drawn the interest of researchers due to a higher rate of the biochemical process, a less variety of fermentation end-products than mesophilic conditions and a reduction of the contamination of hydrogen-consuming microorganisms. Therefore, this study employed the thermophilic bacteria to produce hydrogen under a non-sterile condition.

Currently, as much as 624,000 hectares in Thailand are used for oil palm (*Elaeis guineensis* Jacq.) cultivation, and 81 million cubic meters of old palm trees are generated annually, mainly as a result of the need to replace aging trees after 20-25 years (Department of Commercial Economics, 2010). The oil palm trunks (OPT) comprised of 34.5% cellulose, 31.8% hemicellulose (mainly xylan) and 25.7% lignin (Kelly-Yong et al., 2007). After the hydrolysis of these components, the fermentable sugars are obtained and can be used as the substrate to produce hydrogen by the microbes. Therefore, the bioconversion of the lignocellulosic materials into biofuels such as hydrogen and ethanol could be one of the solutions to make use of these OPT.

In our previous study, the hydrolysis of OPT by microwave assisted sulfuric acid pretreatment was conducted in which a glucose concentration of 8.95 ± 0.99 g/L, xylose concentration of 8.29 ± 0.43 g/L and arabinose concentration of 4.57 ± 0.14 g/L were obtained (Khamtib et al., 2011). Since the hydrolysate of OPT mainly composed of glucose, xylose and arabinose resulting in a high total COD (tCOD) to total nitrogen (TN) ratio (greater than 1000:1) that is not suitable for the production of hydrogen thus a search for the nitrogen source to co-digest with OPT hydrolysate is needed in order to improve a hydrogen production from OPT and to supply a more balance ratio of tCOD/TN. In this study, the slaughterhouse wastewater (SHW) which is rich in nitrogen content from clotted blood was chosen to co-digest with OPT hydrolysate.

In order to enhance a biohydrogen production, the key factors affecting the production of hydrogen must be optimized. Initial cell concentration is one of the key factors that is important in hydrogen production under non-sterile condition. This is because the inoculums augmented into the fermentation system at a low concentration might not be able to compete with the normal flora presence in the fermentation broth under non-sterile condition. In addition, a suitable tCOD/TN ratio could enhance the productivity of hydrogen production (Wang et al., 2011; Radjaram and Saravanane, 2011) because the microorganisms utilized nitrogen for the cell growth as well as hydrogen production. A suitable tCOD/TN for anaerobic digestion is reported to be 33:1 to 45:1 (Sreethawong et al., 2010; Sreela-or et al., 2011). Iron has a great influence on the fermentative hydrogen production since it is the co-factor of hydrogenase enzyme that catalyzes the oxidation of hydrogen (Saraphirom and Reungsang, 2010). However, the hydrogenase enzyme could be inhibited by iron at a high concentration. NaHCO_3 functions as a buffer to counteract with a decrease of pH due to an accumulation of volatile fatty acids (VFAs) in the fermentation broth during fermentative hydrogen production (Boonsayompoo and Reungsang, 2013). In contrast, a high concentration of NaHCO_3 can result in an increase in CO_2 caused by dissolution of carbonate in the system, hence decreasing the hydrogen content in the gas phases (Lin and Lay, 2004a). Magnesium ion is required for the activity of many enzymes such as hexokinase, phosphofructo-1-kinases, glyceraldehydes-3-phosphate dehydrogenases and enolases which functions for stabilization of cell membrane and

cell growth of hydrogen producer such as *Clostridium* sp. (Zhao et al., 2012). Copper can stimulate the microorganisms and enzymes (in glycolysis pathway) activity (Pan et al., 2011). In regard to the above information, it can be seen that in order to achieve a maximum hydrogen production potential (P_s), these key factors must be optimized.

Most of the studies involving the optimization of the process parameters for hydrogen production have been investigated using a one-factor-at-a-time approach (a conventional technique). This method is time-consuming and often leads to misinterpretation of results when interactions between different components are present. Therefore, in order to get rid of these problems and to predict the interaction between the process parameters, the response surface methodology (RSM) have been used.

Accordingly, this study aimed to enhance bio-hydrogen production from OPT hydrolysate by co-digestion with SHW using a statistically based experimental design. Key factors affecting hydrogen production from a co-digestion of OPT hydrolysate and SHW was firstly screened by Plackett-Burman method. Then, the key factors influences the P_s were optimized using RSM with central composite design (CCD). The information from this study would provide the optimum conditions that can maximize P_s from OPT hydrolysate by co-digestion with SHW.

1.2 Thesis objective

The objective of this study is to enhance the bio-hydrogen production from OPT hydrolysate by co-digestion with SHW.

CHAPTER II

LITERATURE REVIEW

2.1 Oil palm trunk (OPT)

Oil palm is one of the important industrial crops in Thailand. In 2010, around 624,000 ha of oil palm planting area distributed over 25 provinces of the country, mainly in the Southern part (Department of Commercial Economics, 2010). In general, the palm starts bearing oil-contained fruits in 3 years after planted and its productivity becomes lower after 20-25 years. Therefore, it is necessary to cut the old palm trees and replant new seedlings at plantation sites for maintaining the oil productivity. In Thailand, most of the old palm trees, 81 million cubic meters per year, are cut and discarded or burnt at the plantation site (Department of Commercial Economics, 2010) which can cause environmental problems as the toxic gasses are emitted.

Oil palm trees are replanted at an average age of 25 years. At the replanting, the trunk ranges between 15-18 m in height and 40-60 cm in diameter, measured 1.5 m above the ground level (Katemanee, 2006). The outer layer of the trunk is the bark, which is about 3.0-3.5 cm thick. The trunk consists of vascular bundles, encrusted in parenchyma ground tissue. The average dry weight per trunk is 600 kg. The average planting density is 138 palms per ha (22 palms per rai) (Chan, et al., 1982). The composition of OPT has been much studied, as shown in Table 2.1.

Table 2.1 Compositions of OPT

Chemical component (wt %)				Researcher
Cellulose	Hemicellulose	Lignin	Ash	
37.14	30.59	22.32	8.56	Rivas et al., 2002
41.00	34.00	16.50	-	Punsuvon et al. 2005
34.50	31.80	25.70	4.30	Khell-Yong et al. 2007
47.50	31.0	18.40	2.69	Chin et al. 2010
42.90	25.60	10.70	1.10	Noparat et al., 2011
45.81	17.74	24.49	11.96	Ang et al., 2013

2.2 Hydrogen production from lignocellulosic hydrolysate

Lignocellulosic materials are an abundant renewable resource worldwide representing an attractive, low-cost feedstock for hydrogen production. However, direct microbial conversion of lignocellulosic materials to hydrogen is inefficient or even impossible due to its complex structure with high crystallinity. Prior to fermentation processes, lignocellulosic materials should be hydrolyzed by diluted acid, alkali, hot water and cellulosic enzymes to generate aqueous-phase monosaccharides-containing hydrolysate (Table 2.2). To date, some studies have been conducted in this field to achieve hydrogen production using hydrolysate of lignocellulosic materials (Cui and Shen, 2012; Chang et al., 2011; Fangkum and Reungsang, 2011), but the hydrogen yield during the processes are found to be relatively low. The reason might lie in the inhibitory effort caused by inhibitors in hydrolysate such as furfural, 5-hydroxy methyl furfural, and acetic acid. Furan derivatives (furfural and 5-hydroxy methyl furfural) have been reported to inhibit growth of microorganisms by reducing enzymatic and biological activities, breaking down DNA and inhibiting protein and RNA synthesis (Liu et al., 2004). Acetic acid is liberated from acetyl groups in hemicellulose fraction (Palmqvist et al., 2000) and can be an inhibitor of microbial growth when present from 4 to 10 g/L. It can enter the cell membrane and decrease intracellular pH, thus affecting the metabolism of the microorganism (van Zyl et al., 1991).

The fermentative hydrogen production from lignocellulosic hydrolysate obtained various pretreatments are presented in Table 2.2.

Table 2.2 Hydrogen production from lignocellulosic hydrolysate

Lignocellulosic materials	Hydrogen producer	Pretreatments	Max. H ₂ yield	References
Grass	<i>Clostridium pasteurianum</i>	Dilute HCl	72.21 mL H ₂ /g TS	Cui and Shen, 2012
Grass	<i>C. pasteurianum</i>	NaOH	19.25 mL H ₂ /g TS	Cui and Shen, 2012
Corn stover	<i>Thermoanaerobacterium thermosaccharolyticum</i> W16	Enzymatic Hydrolysis	108.5 mmol H ₂ / L culture	Ren et al., 2010
Oil palm trunk	<i>T. thermosaccharolyticum</i> W16	Microwave-H ₂ SO ₄	0.71 mol H ₂ / mol sugar	Khamtib et al., 2011
Corn stover	<i>T. thermosaccharolyticum</i> W16	Dilute H ₂ SO ₄	2.24 mol H ₂ / mol sugar	Cao et al., 2010
Corn stover	Activated sludges	Dilute H ₂ SO ₄	5.03 mmol H ₂ /g sugar	Zhang et al., 2011
Corn stalk	Lesser panda manure	Enzymatic Hydrolysis	205.5 mL H ₂ /g-TV S	Xing et al., 2011
Corn stalk	Cow dung compost	Dilute H ₂ SO ₄	144.3 mL H ₂ /g-corn stalk	Song et al., 2012
Oat straw	Anaerobic granular sludges	Dilute HCl	2.9 mol H ₂ /mol hexose	Arriaga et al., 2011
Rice straw	Municipal sewage	Water	24.8 mL H ₂ /g TS	Chen et al., 2012
Rice straw	Municipal sewage	Dilute H ₂ SO ₄	0.191 mmol H ₂ /g-rice straw	Chang et al., 2011
Rice straw	Municipal sewage	Dilute HCl	0.305 mmol H ₂ /g-rice straw	Chang et al., 2011
Sugarcane bagasse	Elephant dung	Dilute H ₂ SO ₄	0.8 mol H ₂ / mol sugar	Fangkum and Reungsang, 2011

2.3 Hydrogen production from co-digestion of substrate

Carbohydrates are the main components of organic wastes from agriculture, food waste and collected organic fraction of municipal solid waste (OFMSW) which have a high C/N ratio ranging from 173:1 to higher than 1,000:1 (Zhang et al. 2008) while the suggested optimum C/N ratio for anaerobic digestion is in the range of 20:1 to 30:1 (Zhou et al., 2013). Therefore, a search for the nitrogen source to co-digest

with carbon rich substrate is needed in order to improve the hydrogen production from carbon rich substrate. The advantages of co-digestion include dilution of potentially toxic compounds, an improved balance of nutrients, and synergistic effects on microorganisms present in the reactions.

Slaughterhouse wastewater, sludge, beverage wastewater and animal manure are high nitrogen content waste. These wastes present a high organic content, high biological oxygen demand (BOD) but low C/N ratio (Callaghan et al. 2002; Edström et al. 2003; Cuetos et al. 2010). High ammonia concentration in animal wastes is considered an inhibitory factor for anaerobic treatments (Nielsen and Angelidaki 2008). This problem is particularly serious when the digesters are fed with wastes rich in protein because, during their fermentation, a significant increase of the ammonia concentration occurs (Chen et al. 2008). In a co-digestion process wastes rich in protein can provide the buffering capacity and a wide range of nutrients, while wastes with high carbon content can balance the C/N ratio for all substrates characterized by a low C/N ratio, decreasing the risk of ammonia inhibition (Hills and Roberts 1981; Hashimoto 1986).

Kim et al. (2012) used the rice straw to co-digest with sewage sludge for hydrogen production. The optimum C/N ratio was found to be 25:1 in which a high and stable hydrogen content of 58% and the maximal HY of 0.74 mmol H₂/g-VS added straw were obtained. A co-digestion of hyacinth with beverage wastewater showed the best hydrogen production performance of the HY (13.65 mL H₂/g feedstock) at a C/N ratio of 42:1 (Lay et al., 2013). Sreela-or et al. (2011) successfully used food waste to co-digest with sludge for hydrogen production. The optimum C/N ratio of 33.1 gave the HY of 102.63 mL H₂/g-VS.

Table 2.3 Hydrogen production from co-digestion of substrate

Co-substrate	Ratio	Hydrogen yield	Reference
Rice straw hydrolysate and sewage sludge	25:1 (C/N)	0.74 mmol H ₂ /g-VS	Kim et al., 2012
Hyacinth and beverage wastewater	42:1 (C/N)	13.65 mL H ₂ /g feedstock	Lay et al., 2013
Food waste and sludge	33.1:1 (C/N)	102.63 mL H ₂ /g- VS	Sreela-or et al., 2011
Cassava stillage and excess sludge	11.9 (C/N)	31.3 mL H ₂ /g-TVS	Wang et al., 2011
Molasses and liquid swine manure	11.6 (C/N)	1.57 mol H ₂ /mol sugar	Wu et al., 2013
Municipal food waste and sewage sludge	31.3:1 (COD/N)	930 mL H ₂ /L feedstock	Zhu et al., 2011
Cassava stillage and excess sludge	54.5:1 (COD/N)	57.8 mL H ₂ /g-TVS	Wang et al., 2013
Cassava stillage and pig Manure	56.7:1 (COD/N)	50 mL H ₂ /g-TVS	Wang et al., 2013
Cassava stillage and cow manure	69.6:1 (COD/N)	45 mL H ₂ /g-TVS	Wang et al., 2013
Cassava stillage and waste activated sludge	54.4:1 (COD/N)	50 mL H ₂ /g-TVS	Wang et al., 2013
Cow manure and waste milk	70:30 (VS/V)	59.5 mL H ₂ /g- VS	Lateef et al., 2012
Municipal food waste and sewage sludge	1:1 (V/V)	112 mL H ₂ /g- VS	Zhu et al., 2008

A co-digestion of municipal food waste and sewage sludge improved a buffering capacity (Zhu et al., 2011). An enhanced pH buffering capacity was identified as the most critical factor influencing HY. According to Zhu et al. (2008), the addition of sewage sludge to food waste provided pH buffering capacity that allowed the fermentation to establish a pH (5.5–6.0) that was contributed to hydrogen production. The response in hydrogen production to pH that was observed in the co-digestion tests was confirmed with separate tests where the pH was buffered using inorganic buffer solutions (K₂HPO₄ and KH₂PO₄). At buffer condition of 0.1 M, the HY from food waste was 90 mL/g VS and this was substantially less than the HY of 250 mL/g VS observed from the mixture of food waste and sewage sludge. This suggests that factors other than pH buffering play an important role in hydrogen production during co-digestion.

CHAPTER III

MATERIALS AND METHODS

3.1 Microorganism

T. thermosaccharolyticum KKU19 (GenBank Accession no. JN020648) was used as the hydrogen producer. It was isolated from hot spring sediment (Betong, Yala Province, Thailand) and identified by 16S rRNA gene sequence in our previous research (Khamtib and Reungsang, 2012). The strain KKU19 was cultivated in basal anaerobic (BA) medium (Angelidaki and Sander, 2004) with 10 g/L glucose as a carbon source. The pH of the medium was adjusted to 6.5 by 5 N HCl or 5 N NaOH. The culture was incubated at 60 °C in water bath for 24 h.

3.2 OPT hydrolysate and SHW

OPT was collected from the farm of Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand. It was chopped by knife, grinded by food blending machine and passed through a 2 mm screen. The grinded OPT was pretreated by microwave assisted sulfuric acid method followed the protocol of Khamtib et al (2011). The resulting OPT hydrolysate was used as the substrate in this study.

The SHW was obtained from Nong Kae Municipal Slaughterhouse, Khon Kaen, Thailand. There are ten pigs and two cows being slaughtered daily. The average volume of wastewater is 2,000 L/day. The SHW contains dilute and clotted blood, fat and some internal organs. The SHW containing debris was blended using a food blender before filtered through a 2 mm screen to remove coarse particle. The filtrate was used as the nitrogen source throughout the study.

The OPT hydrolysate and SHW were stored at -20 °C prior the usage. Table 3.1 summarizes the characteristics of OPT hydrolysate and SHW.

Table 3.1 Characteristics of OPT hydrolysate and SHW

Parameter	OPT hydrolysate	SHW
pH	6.75±0.12	7.12±0.14
Total solid (mg/L)	11,615±360	33,120±259
Volatile solid (mg/L)	9,7800±140	20,900±281
Total COD (mg/L)	21,895±1,213	24,970±2,630
Soluble COD (mg/L)	17,545±780	16,55±2,020
Total carbohydrate (mg/L)	19,218±896	443±81
Total nitrogen (mg/L)	23±1	1,560±210
Alkalinity (mg/L as CaCO ₃)	670±32	2,839±371

3.3 Plackett-Burman design

The Plackett-Burman design was used to screen and identify the key factors used to enhance a hydrogen production from a co-digestion of OPT hydrolysate and SHW by KCU19. The investigated parameters were initial cell concentration (X_1), tCOD/TN ratio (X_2), FeSO₄ concentration (X_3), NaHCO₃ concentration (X_4), MgCl₂ concentration (X_5) and CuSO₄ concentration (X_6). The P_s was selected as the dependent output variables. Based on the Plackett-Burman experimental design, each factor was prepared in two levels: -1 for low level and +1 for high level. Six assigned variables and 5 dummy variables were screened in twelve experiments formulated by Design Expert 7.0.0 Demo version (Stat-Ease Inc., MN, USA). Levels of each factor used in the experimental design and the design matrix are shown in Table 3.2. The effect of each variable was determined by following equation:

$$E_{(X_i)} = \frac{2(\sum M_{i+} - M_{i-})}{N} \quad (1)$$

where, $E_{(X_i)}$ is the concentration effect of the tested variable; M_{i+} and M_{i-} are P_s from runs where the variable (X_i) measured was present at the high and low concentration, respectively, and N is the number of run.

The factors at the 95% level ($p\text{-value} \leq 0.05$) were considered to have a significant effect on P_s and were used in the optimization step by RSM with CCD. In order to approach or locate the area of the optimum, the next step was conducted following the path of steepest ascent.

Table 3.2 Level of the variables studied in Plackett-Burman design

Code	Variable	Low level	High level
		(-1)	(+1)
X ₁	Initial cell concentration (mg dry cell/L)	100	200
X ₂	tCOD/TN ratio	20	40
X ₃	FeSO ₄ concentration (mg/L)	25	50
X ₄	NaHCO ₃ concentration (mg/L)	3000	5000
X ₅	MgCl ₂ concentration (mg/L)	500	1000
X ₆	CuSO ₄ concentration (mg/L)	5	10

3.4 Response surface methodology (RSM)

RSM with CCD was used to evaluate the main and interaction effects of the affected factor obtained from the Plackett-Burman design. The P_s was selected as the dependent output variables and using a predictive polynomial quadratic equation in order to correlate the response variable to the independent variables.

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

where Y_i is the predicted responses; x_i is the parameters; β_0 is a constant; β_i is the linear coefficients; β_{ii} is the squared coefficients; and β_{ij} is the cross-product coefficients.

A differentiation calculation was then employed for predicting the optimum values of different factors giving the maximum P_s . The quality of the fit of quadratic model was expressed by the coefficient of determination, R^2 , and its statically significance was checked by F -test.

3.5 Batch fermentation

Batch fermentation was conducted in 120 mL serum bottles with a 70 mL working volume. The fermentation medium contained OPT hydrolysate, SHW, FeSO₄, NaHCO₃, MgCl₂ and CuSO₄. The inoculum was the cell pellets of the strain KKU19 obtained by centrifugation a suspension of the strain KKU19 at 5000 rpm for 20 min. After obtaining the cell pellets at a designated cell concentration, the cell pellets were re-suspended into the fermentation medium. tCOD/TN ratio, FeSO₄, NaHCO₃, MgCl₂ and CuSO₄ were set according to the design. The medium was adjusted to the initial pH of 6.50 with 3 N HCl or 3 N NaOH. The serum bottles were capped with a rubber stopper, purged with nitrogen gas for 5 min to create anaerobic condition and then incubated at 60 °C in a water bath. During fermentation, the volume of biogas was measured by releasing the pressure in serum bottles using wetted glass syringe. The fermentation process has been continued until biogas is no longer produced. All experiments were performed in triplicates.

3.6 Analytical methods

The cell concentration was determined by measuring the absorbance at 600 nm with a Biophotometer (Eppendorf, Germany). One unit of absorbance was equal to 0.5024 g dry cell/L. The concentrations of volatile fatty acids (VFAs) and alcohols were measured by high performance liquid chromatography (HPLC) (Shimadzu LC-10AD) with an Aminex HPX-87H column followed the method of Fangkum and Reungsang (2011). Carbohydrate concentration was determined by the colorimetric method (Dubois et al., 1956). TN was measured by persulfate method (Ameel et al., 1993). tCOD, soluble COD (sCOD), total solid (TS), total volatile solid (TVS), volatile suspended solid (VSS) and alkalinity were measured using the standard methods (APHA, AWWA, WPCE. 1995). The tCOD/TN ratio was calculated by a following equation:

$$\frac{tCOD}{TN} = \frac{tCOD_{OPT}V_{OPT} + tCOD_{SHW}V_{SHW}}{TN_{OPT}V_{OPT} + TN_{SHW}V_{SHW}} \quad (3)$$

where, tCOD is the total COD of mixed OPT hydrolysate and SHW; TN is the total nitrogen of mixed OPT hydrolysate and SHW; tCOD_{OPT} and TN_{OPT} are total COD and total nitrogen of OPT hydrolysate; tCOD_{SHW} and TN_{SHW} are total COD and total nitrogen of SHW.

The volume of biogas was determined using wetted glass syringes of 20-50 mL (Owen et al., 1979). The biogas produced was analyzed for hydrogen content by gas chromatography (GC) (Shimadzu GC-2014) equipped with a 2 m stainless column packed with Shin carbon (50/80 mesh) followed the method of Fangkum and Reungsang (2011). The cumulative hydrogen production (mL/L substrate) was calculated from the headspace measurement of gas composition and the total volume of hydrogen produced at each time interval was calculated using the mass balance equation (Zheng and Yu, 2005). A modified Gompertz equation was used to fit the cumulative hydrogen production curves to obtain the P_s (mL H₂/L substrate) as explained by Khanal et al. (2004).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Screening of key factors affecting a co-digestion of OPT hydrolysate with SHW

The relative importance of initial cell concentration (X_1), tCOD/TN ratio (X_2), FeSO_4 concentration (X_3), NaHCO_3 concentration (X_4), MgCl_2 concentration (X_5) and CuSO_4 concentration (X_6) for hydrogen production were investigated by Plackett-Burman design. All 12 experiments designed by Design Expert software are listed in Table 4.1. The P_s ranged from 117 ± 13 mL H_2/L substrate to 1577 ± 39 mL H_2/L substrate was obtained. Regression coefficient, *F-values*, *p-values* and effect values (E_{xi}) were analyzed from six factors as showed in Table 4.2. The result of E_{xi} , indicated that the initial cell concentration (X_1), tCOD/TN ratio (X_2), MgCl_2 concentration (X_5) and CuSO_4 concentration (X_6) had a positive effect on P_s . A suitable initial cell concentration of the strain KCU19 is needed to outcompete with a normal flora contained in the SHW. A proper tCOD/TN ratio is required because the nitrogen is needed for cell growth and cell metabolism (Sreethawong et al., 2010; Sreela-or C et al., 2011). The microelements such as magnesium, copper and chloride are important for hydrogen production since they are the cofactor of enzymes in glycolysis pathway e.g. hexokinase, phosphofructo-1-kinases, glyceraldehydes-3-phosphate dehydrogenases and enolases that functions the cell growth of *Clostridium* sp. (Zhao et al., 2012; Pan et al., 2011; Wang et al., 2007). In contrast, FeSO_4 concentration (X_3), NaHCO_3 concentration (X_4) showed a negative effect on P_s . Though the iron is a cofactor of hydrogenase which is important for hydrogen production but a high concentration of iron can inhibit a hydrogen production (Saraphirom and Reungsang, 2010). A dissolution of carbonate at a high concentration of NaHCO_3 can increase CO_2 in the hydrogen production system which in turn decrease the hydrogen content in the gas phase (Lin and Lay, 2004a). High *F-value* and confidence levels greater than 95% ($p\text{-value} \leq 0.05$) of initial cell concentration (X_1), tCOD/TN ratio (X_2) and CuSO_4 concentration (X_6) indicated that

they were statistically significant factors. Therefore, these three variables were selected for further optimization in order to obtain a maximum response in the next step. FeSO_4 concentration (X_3), NaHCO_3 concentration (X_4) and MgCl_2 concentration (X_5) were fixed at 25, 3000 and 1000 mg/L, respectively, in the optimization experiment.

Table 4.1 Plackett-Burman experimental design matrix for evaluating factors influencing P_s

Run	X_1	X_2	X_3	X_4	X_5	X_6	P_s (mL H_2 /L substrate)	
							Observed	Predicted
1	+1	-1	-1	-1	+1	-1	501±24	535
2	-1	-1	-1	+1	-1	+1	117±13	183
3	+1	-1	+1	+1	-1	+1	469±30	497
4	-1	+1	+1	-1	+1	+1	977±55	1024
5	-1	+1	-1	+1	+1	-1	792±27	877
6	-1	-1	+1	-1	+1	+1	264±13	217
7	+1	+1	-1	-1	-1	+1	1419±40	1454
8	+1	+1	-1	+1	+1	+1	1577±39	1448
9	+1	+1	+1	-1	-1	-1	1175±48	1197
10	-1	-1	-1	-1	-1	-1	169±21	77
11	-1	+1	+1	+1	-1	-1	792±30	732
12	+1	-1	+1	+1	+1	-1	375±28	385

Table 4.2 Coefficient, estimated effect and corresponding F -value and p -value in the Plackett-Burman

Code	Coefficient	Effect (E_{xi})	F -value	Prob > F
X_1	196.10	392.20	32.67	0.0046
X_2	407.57	815.13	141.12	0.0003
X_3	-43.50	-95.30	1.93	0.2372
X_4	-31.83	-69.74	1.03	0.3669
X_5	33.23	66.47	0.94	0.3876
X_6	89.40	178.80	6.89	0.0497

4.2 The path of steepest ascent

The path of the Steepest Ascent was used to find the proper directions of changing variables. The directions of changing the initial cell concentration (X_1), tCOD/TN ratio (X_2) and CuSO_4 concentration (X_6) were tabulated in Table 4.3. Maximum P_s of 2307 ± 91 mL H_2 /L substrate was obtained at 220 mg dry cell/L of initial cell concentration, 50 of tCOD/TN ratio and 15.00 mg/L of CuSO_4 concentration (Run 2). Thus this condition was used as the central point in the optimization experiment using RSM with CCD.

Table 4.3 Experimental design and results of the path of steepest ascent

Run	Initial cell concentration (X_1)(mg dry cell/L)	tCOD/TN ratio (X_2)	CuSO_4 concentration (X_6)(mg/L)	P_s (mL H_2 /L substrate)
1	200	40	10	1620 \pm 78
2	220	50	15	2307 \pm 91
3	240	60	20	1582 \pm 60
4	260	70	25	1651 \pm 54
5	280	80	30	1232 \pm 21

4.3 Effect of the key factors on a co-digestion of OPT hydrolysate with SHW

Effects of inoculum concentration (X_1), C/N ratio (X_2) and CuSO_4 concentration (X_6) on P_s (Y_{Ps}) were investigated during the hydrogen fermentation. The maximum P_s of 2655 ± 82 mL H_2 /L substrate was obtained in run 7 (initial cell concentration of 220 g dry cell/L, tCOD/TN ratio of 50 and CuSO_4 concentration of 15 mg/L) while the lowest P_s of 1230 ± 39 mL H_2 /L substrate was found in run 16 (initial cell concentration of 186.4 g dry cell/L, tCOD/TN ratio of 50 and CuSO_4 concentration of 15 mg/L). The multiple regression analysis of experimental data (Table 4.4) resulted in the quadratic equation (Eq. 4) as follow:

$$Y_{Ps} = 2546.79 + 140.75X_1 - 33.43X_2 - 90.23X_6 + 12.51X_1X_2 - 4.94X_1X_6 - 77.54X_2X_6 - 380.54 X_1^2 - 205.35X_2^2 - 135.53X_6^2 \quad (4)$$

Table 4.4 Central composite experiment design matrix defining initial cell concentration (X_1), tCOD/TN ratio (X_2) and CuSO_4 concentration (X_6) for optimizing the hydrogen production and the corresponding experimental result

Run	Initial cell concentration (X_1) (mg dry cell/L)		tCOD/TN ratio (X_2)		CuSO ₄ concentration (X_6) (mg/L)		P _s (mL H ₂ /L substrate)	
	Actual	Code	Actual	Code	Actual	Code	Observed	Predicted
	1	200	-1	60.00	+1	10.00	-1	1729±54
2	220	0	50.00	0	15.00	0	2484±68	2547
3	200	-1	60.00	+1	20.00	+1	1511±50	1476
4	220	0	50.00	0	15.00	0	2550±70	2547
5	240	+1	40.00	-1	20.00	+1	2036±69	1969
6	220	0	50.00	0	10.00	0	2569±72	2547
7	220	0	50.00	0	15.00	0	2655±82	2547
8	220	0	66.82	+1.682	15.00	0	1960±46	1910
9	220	0	50.00	0	23.41	+1.68	1978±21	2012
10	253.6	+1.682	50.00	0	15.00	0	1719±35	1707
11	240	+1	60.00	+1	20.00	+1	1725±29	1773
12	220	0	50.00	0	15.00	0	2452±68	2547
13	200	-1	40.00	-1	10.00	-1	1781±80	1738
14	200	-1	40.00	-1	20.00	+1	1721±35	1723
15	240	+1	60.00	+1	10.00	-1	2115±56	2118
16	186.4	-1.682	50.00	0	15.00	0	1230±39	1234
17	220	0	50.00	0	6.59	-1.682	2357±56	2315
18	220	0	50.00	0	15.00	0	2569±70	2547
19	220	0	33.18	-1.682	15.00	0	1980±50	2022
20	240	+1	40.00	-1	10.00	-1	1964±44	2005

The analysis of variance (ANOVA) of the model indicated that the model significantly represents the experimental data (p -value < 0.0001). A high determination coefficient (R^2) of 0.9840 and the adjusted determination coefficient (adjusted R^2) of 0.9695 suggested that the model can explain 97-98% variability of the response variable. In addition, lack of fit of the model was insignificant (p -value = 0.5314) indicating that Eq. (4) properly described the P_s (Table 4.5).

The response surface plot drawn based on the graphical representations of quadratic regression equation Eq. (4) are shown in Figure 4.1A-C. The P_s increased with an increase in initial cell concentration (X_1) from 200 to 223.78 mg dry cell/L

and then decreased with a further increase in initial cell concentration greater than 223.78 mg dry cell/L (Figure 4.1A and B). Under a non-sterile condition, the indigenous microflora present in the substrate i.e. mixed OPT hydrolysate and SHW might outcompete the strain KKU19 at low initial cell concentration. An increase in initial cell concentration of the strain KKU19 to a concentration greater than the optimum value could contribute to a production and accumulation of fermentation metabolites i.e., VFAs causing a rapid drop of pH in the fermentation broth which could inhibit the growth of hydrogen producers (Fan et al., 2006). Moreover, at high accumulation of VFAs in the fermentation broth, the ionic strength of solution will increase, resulting in a switch from hydrogen and acids productions to solvent production (Chong et al., 2009). Therefore, the initial cell concentration should be compatible with the available substrate for maximizing the bacterial activity.

An increase in tCOD/TN ratio (X_2) from 40 to 49.87 resulted in an increase in P_s (Figure 4.1A and C). However, a further increase in tCOD/TN ratio (X_2) above 49.87 caused a reduction in P_s . At optimum tCOD/TN ratio, the microbes use nitrogen from the substrate to enhance the cell growth and hydrogen production (Sreethawong et al., 2010; Sreela-or et al., 2011). However, the low tCOD/TN ratio (excess N content) could decrease hydrogen production because ammonium ions generated from protein and nitrogen complex degradation can inhibit cell growth and hydrogen production. A high tCOD/TN ratio (low N content) could decrease hydrogen production efficiency because nitrogen is deficient for cell growth (Lin and Lay, 2004b). The optimum tCOD/TN for hydrogen production from co-digestion was reported to be in the range of 33:1 to 45:1 (Sreethawong et al., 2010; Sreela-or et al., 2011) depending on types of substrate and microorganisms. Our results showed a similar trend in which the optimum tCOD/TN ratio was 49.87.

The effect of CuSO_4 concentration (X_6) was similar to the effect of inoculum concentration (X_1) and tCOD/TN ratio (X_2) on P_s in which the P_s increased with an increase CuSO_4 concentration (X_6) from 10 to 13.33 mg/L and then decreased when the CuSO_4 concentration (X_6) was further increased above 13.33 mg/L (Figure 4.1B and C). Cu^{2+} at a concentration of 8 mg/L was reported to have a stimulating effect on the activity of microorganisms (Altas, 2009) and on the activation or function of microbial enzyme and coenzyme such as glyceraldehydes-3-

phosphate dehydrogenases, and hydrogenase (Pan et al., 2011). However, at as high Cu^{2+} concentration as 30 mg/L, the hydrogen production could be over 50% decreased because Cu^{2+} is toxic to the microbial cells (Li and Fang, 2007).

Table 4.5 ANOVA for the model regression representing P_s in CCD

Source	Sum of squares	df	Mean of square	F- value	Probe > F
Model	3042260	9	338028.9	68.16841	< 0.0001
X_1	270552.2	1	270552.2	54.56076	< 0.0001
X_2	15260.05	1	15260.05	3.077409	0.1099
X_6	111198.2	1	111198.2	22.42473	0.0008
$X_1 X_2$	1252.501	1	1252.501	0.252585	0.6261
$X_1 X_6$	195.0313	1	195.0313	0.039331	0.8468
$X_2 X_6$	48096.51	1	48096.51	9.699356	0.0110
X_1^2	2086891	1	2086891	420.8518	< 0.0001
X_2^2	607720.2	1	607720.2	122.5556	< 0.0001
X_6^2	264696	1	264696	53.37978	< 0.0001
Residual	49587.32	10	4958.732		
Lack of Fit	23876.79	5	4775.358	0.928677	0.5314
Cor Total	3091848	19			
Coefficient of determination (R^2) = 0.9840					
Adjusted determination coefficient (adj R^2) = 0.9695					

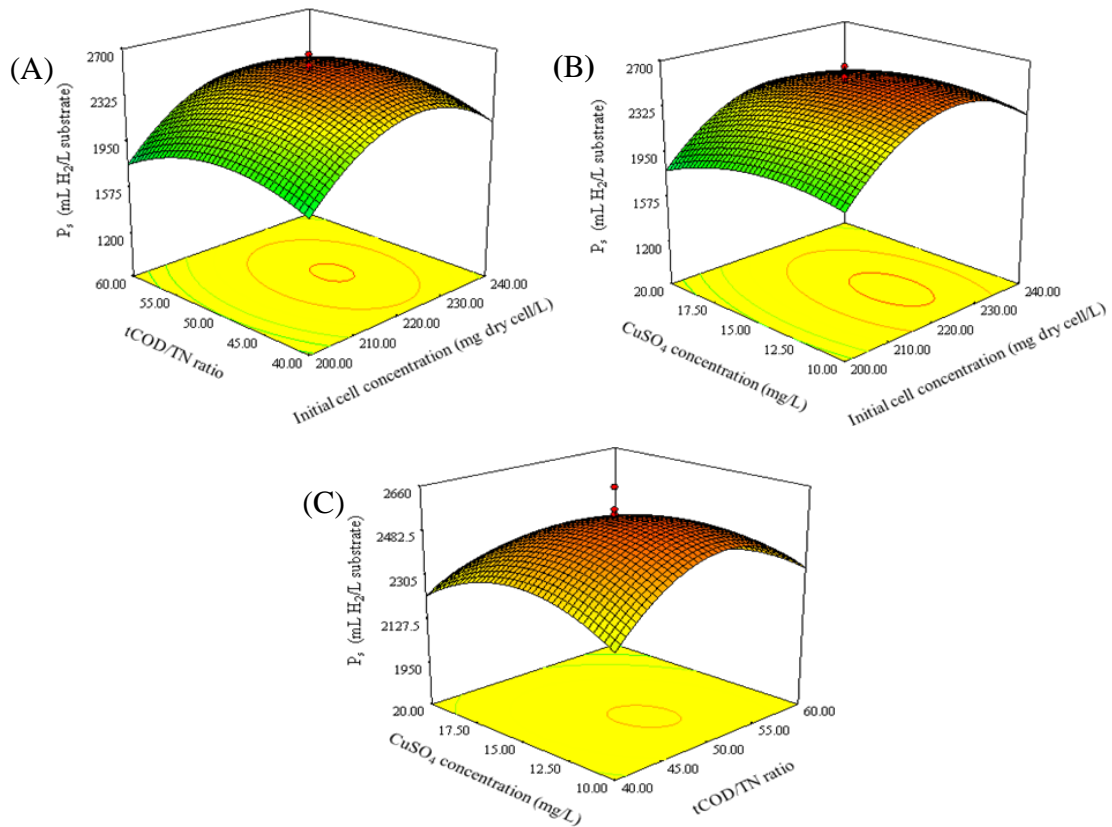


Figure 4.1 Response surface plots showing the effect of initial cell concentration (X_1) and tCOD/TN ratio (X_2) (A); the effect of initial cell concentration (X_1) and CuSO_4 concentration (X_6) (B); the effect of tCOD/TN ratio (X_2) and CuSO_4 concentration (X_6) (C) on the hydrogen production.

4.4 Optimization and confirmation of the experiment

The predicted optimum conditions for a maximum P_s were an initial cell concentration of 223.78 mg dry cell/L, a tCOD/TN ratio of 49.87 and a CuSO_4 concentration of 13.33 mg/L, in which a maximum predicted P_s of 2575 mL H_2 /L substrate was obtained. To validate the adequacy of the model, five experiments were conducted under the optimum, high (run 11), medium (run 6) and worst (run 13) conditions (Table 4.6) as well as the control which was the treatment that only the OPT hydrolysate was used as the substrate to produce hydrogen by the strain KKU19.

Under the optimum conditions, the observed P_s of 2604 ± 86 mL H_2 /L substrate was obtained which is only 1.10 % different from the maximum predicted P_s (2575 mL H_2 /L substrate). The result indicated that a statistical experimental design i.e.

RSM with CCD is useful tool to optimize the fermentative hydrogen production condition. At the optimum condition, the main soluble metabolite products (SMPs) were butyric acid (48.81%), acetic acid (24.55%) and ethanol (20.83%) (Table 4.6). The presence of high amount of butyrate and acetate in this study indicated that the hydrogen fermentation was butyrate-acetate type fermentation.

The P_s obtained at optimum conditions (2604 mL H_2/L substrate) was approximately 1.14 times higher than the results obtained when only OPT hydrolysate (control) was used as the substrate to produce hydrogen by the strain KKU19 (2289 mL H_2/L substrate). Therefore, the results from this study implied that the use of SHW to co-digest with the OPT hydrolysate could enhance the hydrogen production by the strain KKU19.

Comparisons of the results on P_s obtained in this study to the other literatures search are showed in Table 4.7. The P_s obtained in this study (2604 mL H_2/L substrate) was approximately 1.34 times higher than the results obtained when only OPT hydrolysate was used as the substrate to produce hydrogen by hot spring enriched culture (1948 mL H_2/L substrate) (Khamtib et al., 2011). However, the P_s from our co-digestion study is lower than the studies of Hniman et al. (2011) who used only OPT hydrolysate as the substrate to produce hydrogen by hot spring enriched culture. The differences in the hydrogen production might be due to the difference in the types of microorganisms, hydrolysis conditions, compositions of the hydrolysate and fermentation conditions.

Table 4.6 Confirmation hydrogen production experiment

Run	Conditions	Initial cell concentration (X ₁) (mg dry cell/L)	tCOD/TN ratio (X ₂)	CuSO ₄ concentration (X ₆) (mg/L)	Final pH	Substrate consumed (g-COD/L)	P _s (mL H ₂ /L)		HY (mL H ₂ /g-COD _{consumed})	SMPs (g/L)					
							Observed	Predicted		HFr	HAc	HPr	HBu	HLc	EtOH
-	Optimum	223.78	49.87	13.33	4.12±0.21	4.86±0.17	2604±123	2579	535.8±12.7	0.32±0.02	1.65±0.06	0.00±0.00	3.28±0.24	0.07±0.02	1.40±0.15
11	High	240	60	20	4.19±0.17	4.29±0.18	1930±102	1768	449.9±11.2	0.03±0.01	1.22±0.11	0.01±0.00	3.15±0.27	0.15±0.03	1.83±0.08
6	Medium	220	50	15	4.07±0.18	4.61±0.12	2565±116	2548	556.4±11.8	0.17±0.02	1.52±0.14	0.00±0.00	2.92±0.18	0.23±0.03	1.36±0.12
13	Worst	200	40	10	3.97±0.22	4.01±0.21	1740±74	1763	433.9±8.2	0.06±0.01	0.92±0.08	0.00±0.00	2.51±0.21	0.62±0.06	1.28±0.06
-	Control*	223.78	950*	13.33	3.85±0.34	4.73±0.26	2289±126	-	483.9±12.3	0.21±0.02	2.39±0.33	0.00±0.00	2.52±0.29	0.05±0.01	1.36±0.14

SMPs: Soluble metabolite products (g/L)

HFr: formic acid; HAc: acetic acid; HPr: propionic acid; HBu: Butyric acid; HLac: lactic acid; EtOH: ethanol.

P_s: hydrogen production potential (mL H₂/L substrate)

HY: hydrogen yield (mL H₂/g-COD_{consumed})

* Control: The treatment that only the OPT hydrolysate was used as the substrate to produce hydrogen by the strain KKU19.

Table 4.7 Comparisons hydrogen production using the hydrolysate of lignocellulosic materials by thermophilic hydrogen producer

Microorganism	Substrate	pH	Temperature (°C)	Initial substrate concentration (g-COD/L)	P _s (mL H ₂ /L substrate)	References
Hot spring enriched culture	OPT hydrolysate	6.03	55	22.07	1948	Khamtib et al., 2011
Hot spring enriched culture	OPT hydrolysate	NA	60	14.88	3048	Hniman et al.,
<i>T. thermosaccharolyticum</i> W16	Corn stover hydrolysate	6.50	60	10.00	3279	Ren et al., 2008
<i>T. thermosaccharolyticum</i> KKU19	Cellulose fraction of sweet sorghum bagasse	6.50	55	10.41	2186	Boonsayompoo and Reungsang, 2013
<i>T. thermosaccharolyticum</i> KKU19	Co-digestion of OPT hydrolysate and SHW	6.50	60	22.76	2604	This study

CHAPTER V

CONCLUSIONS

This study successfully enhanced hydrogen production by co-digesting the OPT hydrolysate with SHW. The optimum conditions for hydrogen production from co-digestion of OPT hydrolysate with SHW were identified as initial cell concentration of 223.78 mg dry cell/L, tCOD/TN ratio of 49.87 and CuSO_4 concentration of 13.33 mg/L. The P_s from the confirmation experiment under optimum conditions was 2604 ± 86 mL H_2 /L substrate which was only 1.10 % different from the predicted P_s (2575 mL H_2 /L substrate). Main SMPs were butyric acid (48.81%) and acetic acid (24.55%). In addition, co-digestion can enhance hydrogen production approximately 1.14 times than OPT hydrolysate alone. Our results indicated that SHW can serve as nitrogen source for bio-hydrogen production.

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