CHAPTER III

OPTIMIZATION OF BIOHYDROGEN PRODUCTION FROM SWEET SORGHUM SYRUP USING STATISTICAL METHODS

3.1 Introduction

Depletion of fossil fuel has brought people's attention into the findings of renewable energy resource in which biomass both from crops and wastes are the most potential and promising ones. Renewable energy from biomass includes ethanol e.g. from corn [1], sugarcane [2], sweet sorghum juice [3] and cassava [4]; biodiesel e.g. from palm oil [5] and used oil [6]; methane e.g. from waste [7], manure [8]; and hydrogen e.g. from sweet sorghum [9], sugarcane [10], sweet potato [11], cassava wastewater [12], and activated sludge [13]. However, there are concerns on using crop as an energy plant or food plant especially on producing ethanol which is mainly be produced from crops. Thus, people have paid attention into finding energy plant as well as waste as a feedstock for producing the renewable energy. Methane and hydrogen are renewable energy but hydrogen has more advantages than methane due to its cleaness, efficient and non-polluting characteristics [14]. When hydrogen is combusted with oxygen, water is the by product so no greenhouse gas is produced unlike methane. It is 50% more efficient than gasoline which energy is 2.75-folds greater than hydrocarbon fuel sources [15]. Hydrogen can be produced by physiochemical and biological methods. Physicochemical method includes water electrolysis, steam reforming of methane and hydrocarbons. This method is energy intensive process requiring high temperature (>850 °C) [16]. Biological method is attractive because it is energy saving process as compared to chemical process [14, 17, 18]. Biological hydrogen production by microorganisms falls into two main categories i.e., light fermentation process by photosynthetic bacteria and algae and dark fermentation by anaerobic bacteria [19]. Dark fermentation has shown to have a

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great potential for development as a practical bio-hydrogen production system [20]. Dark fermentation can use biomass such as energy crops, agricultural or industrial wastes and solid wastes as substrate to produce hydrogen [21]. Different groups of bacteria are known to be responsible for hydrogen production by dark fermentation such as *Enterobacter*, *Bacillus* and *Clostridium* [22]. Not only pure culture but also mixed anaerobic microorganisms obtained from sewage or wastewater treatment plant sludge are able to produce hydrogen via the dark fermentation process [22, 23].

Sweet sorghum (Sorghum bicolor var. Keller) is gaining an attention as a potential alternative feedstock for energy and industry due to its high biomass yield and sugar [24]. In Thailand, sweet sorghum can grow in almost every geographic part. Sweet sorghum can be harvested within 100-120 days while sugarcane takes at least one year per crop and its production is not much different than sugarcane in which the average production of sweet sorghum is 31.25-37.5 tons/ha while production of sugarcane is 56.25-62.50 tons/ha [25]. The Keller variety has high sugar productivity depending on the harvest time. Fresh biomass and fresh stalk yield 8-11.5% and 9-14.5% of total sugar content, respectively [26], which mainly contains sucrose, fructose and glucose. These sugars are good substrates for ethanol and hydrogen productions. *Saccharomyces cerivisiae* and other species of the genus *Saccharomyces* [27] can direct ferment sugar in sweet sorghum juice to produce ethanol [28]. Mixed cultures can produce hydrogen from sweet sorghum extracts with the maximum yield of 0.86 mol H_2/mol glucose consumed [9]. Moreover, pure culture strain *Ruminococcus albus* can produce hydrogen from sugars and sweet sorghum biomass [29].

Biohydrogen production using dark fermentation is greatly influenced by environmental factors such as temperature, pH, nutrient addition, ferrous iron and substrate concentration [30]. In order to efficiently produce hydrogen there is a need to optimize these environmental factors. However, it is laborious and time consuming to perform the optimization by a conventional technique or known as "a one factor at a time" method [31]. A statistical experimental design by the Plackett-Burman and response surface methodology (RSM) can eliminate this limitation [32]. It is not only a time saving method but also can minimize the error in determining the effects of

parameters as well as be able to demonstrate the interactive effects among the tested variables [33]. Statistical optimization design on biohydrogen production has recently been reported in literatures [34, 30, 35, 36, 37]. For instance, Mu et al. [34] studied factors affecting bio-hydrogen production by enriched anaerobic cultures from sucrose using central composite design. Lee et al. [30] investigated the effects of temperature, pH and starch concentration on fermentative hydrogen production from starch by mixed anaerobic microflora using RSM. Pan et al. [35] investigated process parameters on bio-hydrogen production from glucose by *Clostridium* sp. Fanp2 using Plackett-Burman design and Box-Behnken design. O-Thong et al. [37] investigated factors affecting hydrogen production from palm oil mill effluent (POME) under thermophilic condition using a central composite design and RSM. Although many studies have been done on the effect of environmental factors on hydrogen production from various kinds of synthetic substrates and wastes but the information on the statistically optimization of environmental factors on biohydrogen production from sweet sorghum by mixed cultures are still lacking.

Therefore, this present study aims to optimize the environmental factors affecting hydrogen production from sweet sorghum syrup by anaerobic mixed cultures. The Plackett-Burman design is used to screen the significant variables and then the Box-Behnken design of RSM is used to optimize the levels of the screened variables. It is hope that the results from this study will provide the optimum condition that can improve hydrogen yield from sweet sorghum syrup.

3.2 Materials and Methods

3.2.1 Seed and inoculum preparation

The anaerobic seed sludge was obtained from a full scale anaerobic digester of Upflow Anaerobic Sludge Blanket (UASB) reactor of the brewery company in Khon Kaen, Thailand. The UASB is used to produce methane from the wastewater of beer production process. Prior to use, the anaerobic sludge was heated at 105 °C for 2 hours to inactivate methanogenic bacteria and then cooled at room temperature in dessicator. For inoculum preparation, the culture was cultivated in 20 g/L total sugar of sweet sorghum syrup as a carbon source with the supplementation of nutrient solution [38] at a rate of 0.5 mL/L. The composition of nutrient solution

was composed as follows 200 g/L NH_4HCO_3, 100 g/L KH_2PO_4, 10 g/L $MgSO_4.7H_2O$, 1 g/L NaCl, 1 g/L $Na_2MoO_4.2H_2O$, 1 g/L $CaCl_2.2H_2O$ and 0.278 g/L $FeCl_2$. The culture was shaken at 150 rpm for 24 hours before used as the inoculum in batch experiment.

3.2.2 Sweet sorghum syrup

Sweet sorghum (Sorghum bicolor var. Keller) used in this study was obtained from the field experiment of Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand. Sweet sorghum syrup was juice extracted from sorghum cane and concentrated by heating. Total sugar of sweet sorghum syrup was 75-80 °Brix determined by a hand refractometer (HRB-32 ATC). Sweet sorghum syrup was sterilized at 110 °C for 28 min to prevent the contamination and diluted with distilled water to a designed concentration before used as substrate for hydrogen production.

3.2.3 Biohydrogen production

Biohydrogen production experiment was conducted in 120 mL serum bottles with a working volume of 70 mL. The hydrogen production medium contained 10% inoculum (v/v) aand different concentrations of variables according to the design. Then serum bottles were flushed with argon gas to remove oxygen in headspace of bottles and create the anaerobic condition and then capped with rubber stopper. The bottles were incubated at room temperature and operated in an orbital shaker with a rotation speed of 150 rpm to provide better contact among substrates. At each time interval, the total gas volume was measured by releasing the pressure in the bottles using wetted glass syringe [39]. All treatments were conducted in four replications. The anaerobic digestion was continued until biogas volume could not be measured.

3.2.4 Analytical method

Biogas composition was measured by a gas chromatograph (GC-2014, Shimadzu) equipped with a thermal conductivity detector (TCD) and 2 m stainless column packed with Unibeads C (60/80 mesh). The operational temperatures of the injection port, the column oven and the detector were 150, 145 and 150 °C, respectively. Argon was used as the carrier gas at a flow rate of 25 mL/min. For volatile fatty acids (VFAs), acetone and alcohols analysis, the liquid samples were first centrifuged at 6,000 rpm for 10 min, acidified by 0.2N oxalic acid and filtered

through 0.45 μm cellulose acetate membrane. The same GC model with a flame ionization detector (FID) and a 30 m x 0.25 mm x 0.25 μm capillary column (Stabiwax) was used. The temperature of the injector and detector were 250 °C. The initial temperature of column oven was 50 °C for 2 min followed with a ramp of 15 °C/min for 12.6 min and to final temperature of 240 °C for 1 min. Helium was used as carrier gas with a flow rate of 66 mL/min.

Total sugar, volatile suspended solid (VSS) and total solid (TS) were measured according to the procedures described in standard methods [40]. Hydrogen gas production was calculated from the headspace measurement of gas composition and the total volume of hydrogen produced, at each time interval, using the mass balance equation (Eq. 1) [41]:

$$V_{H,i} = V_{H,i-1} + C_{H,i}\left(V_{G,i} - V_{G,i-1}\right) + V_{H,0}\left(C_{H,i} - C_{H,i-1}\right) \quad (1)$$

where $V_{H,i}$ and $V_{H,i-1}$ are the cumulative hydrogen gas volumes at the current (i) and previous time interval $(i-1)$, respectively; $V_{G,i}$ and $V_{G,i-1}$ are total biogas volume at the current and previous time interval; $C_{H,i}$ and $C_{H,i-1}$ are the fraction of hydrogen gas in the headspace at the current and previous time interval; V_H is the volume of headspace of serum bottles (50 mL).

3.2.5 Kinetic modeling

A modified Gompertz equation (Eq. 2) was used to fit the cumulative hydrogen production curves to obtain the hydrogen production potential (P_s), the hydrogen production rate (R) and lag phase (λ).

$$H = P\exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t) + 1\right]\right\} \quad (2)$$

Where, H represents the cumulative volume of hydrogen produced (mL), P_s the hydrogen production potential (mL), R_m the maximum production rate (mL/h), λ the lag-phase time (h), t the incubation time (h), and e is 2.718281828. Parameters (P_s, R_m and λ) were estimated using the solver function in Microsoft Excel version 5.0 (Microsoft, Inc.) as explained by Khanal et al. [42]. In this study

R_m is expressed as mL of H_2/(L of medium/h), the specific hydrogen production potential P_s is defined as mL H_2/L medium.

3.2.6 Optimization procedure

3.2.6.1 Plackett-Burman design

This study used the Placket-Burman design to screen and identify significant variables for hydrogen production from sweet sorghum syrup by mixed cultures. Culture parameters investigated were total sugar concentration, initial pH, nutrient addition, iron (II) sulphate ($FeSO_4$), peptone and sodium bicarbonate ($NaHCO_3$) concentration [43]. Composition of nutrient solution used in screening significant variables for hydrogen production contains 40 mg/L $MgCl_2.6H_2O$, 0.1 mg/L $CoCl_2.6H_2O$, 10 mg/L $CaCl_2.2H_2O$, 2.5 mg/L $NiCl_2.6H_2O$, 2.5 mg/L $MnCl_2.6H_2O$, 12.5 mg/L KI, 1000 mg/L NaCl, 0.1 mg/L $ZnCl_2$, 2.5 mg/L $MnSO_4.4H_2O$, 5 mg/L $CuSO_4.5H_2O$ and 0.1 mg/L $Na_2MoO_4.2H_2O$ (modified from Lin and Lay, 2005) [38].

Placket-Burman experimental design [43] is based on the first order model:

$$Y = \beta_0 + \sum \beta_i X_i \tag{3}$$

where, Y is the response, β_0 is the model intercept and β_i is the linear coefficient, and X_i is the level of the independent variable. Based on the Placket-Burman experimental design, each factor was prepared in two levels: -1 for low level and +1 for high level. Six assigned variables and three unassigned variables or so-called the dummy were screened in twelve experimental designs. Levels of each factor used in the experimental design and the design matrix were shown in Table 1 and Table 2, respectively.

The effect of each variable was determined by following equation:

$$E_{(Xi)} = \frac{2\left(\sum M_{i+} - M_{i-}\right)}{N} \tag{4}$$

where, E_{Xi} 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 (12). The statistical software package Design-Expert 7.0.0, Stat-Ease, Inc., Minneapolis, MN, USA was used as a tool for statistical analysis.

Table 1 Level of variables, estimated effect, regression coefficient and corresponding F and P values for P_s in Plackett-Burman design.

Code	Variable	Low level (-1)	High level (+1)	Coefficient	Effect (E_{Xi})	F-value	P-value $Prob>F$
X_1	Total sugar (g/L)	5	15	547.28	1094.56	63.51	0.0005
X_2	Initial pH	5.0	7.0	-107.47	-214.96	2.45	0.1784
X_3	Nutrient (mL/L)	1	10	66.81	133.63	0.95	0.3753
X_4	$FeSO_4$ (g/L)	0.2	0.6	119.29	238.57	3.02	0.1429
X_5	Peptone (g/L)	1	3	-30.81	-61.63	0.20	0.6724
X_6	$NaHCO_3$ (g/L)	2.5	6	-57.00	-114.00	0.69	0.4443

Table 2 Plackett-Burman experimental design matrix for evaluating factors influencing P_s from sweet sorghum syrup by mixed cultures.

Run #	Experimental values						P_s (mL H_2/L)	
	X_1	X_2	X_3	X_4	X_5	X_6	Observed [a]	Predicted
1	+1	+1	-1	+1	+1	+1	1858.28	1791.47
2	-1	+1	+1	-1	+1	+1	297.16	591.97
3	+1	-1	+1	+1	-1	+1	1985.72	2201.67
4	-1	+1	-1	+1	+1	-1	743.44	810.91
5	-1	-1	+1	-1	+1	+1	1101.72	806.91
6	-1	-1	-1	+1	-1	+1	1021.16	973.43
7	+1	-1	-1	-1	+1	-1	1860.56	1881.85
8	+1	+1	-1	-1	-1	+1	1716	1614.53
9	+1	+1	+1	-1	-1	-1	1909.16	1862.15
10	-1	+1	+1	+1	-1	-1	1153.16	1006.17
11	+1	-1	+1	+1	+1	-1	2276	2254.05
12	-1	-1	-1	-1	-1	-1	721.72	848.91

[a] The observed values of P_s were the mean values of four replications.

In order to approach the area of the optimum, the next experiment was carried out along the path of steepest ascent. The direction of the maximum increase in P_s was yielded by the gradient of the regressed polynomial.

3.2.6.2 Box-Behnken design

A three-variable Box-Behnken design [35] with four replicates at the center point was applied in order to optimize the significant factors enhanced hydrogen production. For statistical calculations, the relation between the codes values and actual values are described by equation as follows:

$$X_i = \frac{(A_i - A_0)}{\Delta A} \tag{5}$$

where X_i is a coded value of the variable; A_i the actual value of variable; A_0 the actual value of the A_i at the centre point; and ΔA the step change of variable. Table 3 illustrated the levels of the variables and the experimental design.

In order to predict the optimal point, a second-order polynomial function was fitted to correlate relationship between variables and response (P_s) using a Quadratic equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \tag{6}$$

where, Y is the predicted response; β_0 a constant; β_i the linear coefficients; β_{ii} the squared coefficients; and β_{ij} the cross-product coefficients.

The software Design-Expert 7.0.0 was used for the regression and graphical analysis of the experimental data obtained. A differentiation calculation was then employed for predicting the optimum values of different factors for 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 using Design-Expert 7.0.0, Stat-Ease, Inc., Minneapolis, MN, USA.

Table 3 The Box-Behnken experimental design with three independent variables.

Run #	Variables/levels						P_s (mL H_2/L)	
	Total sugar		Initial pH		$FeSO_4$			
	Actual (g/L)	Code	Actual	Code	Actual (g/L)	Code	Observed	Predicted
1	20	-1	4.0	-1	1.45	0	4946.73	5084.97
2	30	+1	4.0	-1	1.45	0	5973.43	5890.79
3	20	-1	5.5	+1	1.45	0	5926.14	6008.78
4	30	+1	5.5	+1	1.45	0	5161	5022.72
5	20	-1	4.75	0	0.9	-1	5694.86	5462.74
6	30	+1	4.75	0	0.9	-1	5227.90	5216.66
7	20	-1	4.75	0	2	+1	6055.14	6066.39
8	30	+1	4.75	0	2	+1	5900.14	6132.27
9	25	0	5.5	+1	0.9	-1	4680	4773.89
10	25	0	5.5	+1	0.9	-1	4289	4438.48
11	25	0	4.0	-1	2	+1	5319.71	5170.23
12	25	0	5.5	+1	2	+1	5655.29	5561.41
13	25	0	4.75	0	1.45	0	6807.57	6896.63
14	25	0	4.75	0	1.45	0	6937.86	6896.63
15	25	0	4.75	0	1.45	0	6890	6896.63
16	25	0	4.75	0	1.45	0	6992.71	6896.63
17	25	0	4.75	0	1.45	0	6855	6896.63

3.2 Results and Discussion

3.3.1 Screening of significant variables for hydrogen production by anaerobic mixed cultures

The main effect of each variable upon P_s was shown in Table 1. The effect was estimated as the difference between both averages of measurements made at the high level (+1) and at the low level (-1) of that factor. The value is used to indicate the relative importance of total sugar concentration, initial pH, nutrient solution, $FeSO_4$, peptone and $NaHCO_3$ concentration on hydrogen production from sweet sorghum syrup by mixed cultures. If the influence of the variable on P_s is greater at a high level, the sign of the effect E_{X_i} of the tested variable is positive; if it

is greater at a low level, the sign of the effect E_{Xi} of the tested variable is negative. Results from Table 1 indicated that high level of X_1 (total sugar concentration), X_3 (nutrient solution) and X_4 ($FeSO_4$) enhanced hydrogen production. In contrast, low level of X_2 (initial pH), X_5 (peptone) and X_6 ($NaHCO_3$) resulted in high P_s. The probability value of less than 0.05 was found with a total sugar concentration ($P < 0.0005$) and high positive probability values were found with ferrous sulphate ($P = 0.1429$), and initial pH ($P = 0.1784$) (Table 1), which suggested a significant effect of these variables on hydrogen production. Therefore, these three variables were selected for further optimization experiment through RSM.

3.3.2 The path of steepest ascent

This step is used to attain the proper direction of changing variables, i.e., increasing the total sugar and $FeSO_4$ concentrations while decreasing the initial pH to improve the hydrogen production potential. The directions of changing the three variables were shown in Table 4. Results indicated that Run 4 (Table 4) gave the maximum hydrogen production potential of 4,353 mL H_2/L thus this condition was chosen in the next optimization step.

Table 4 Experimental design and results of the path of steepest ascent.

Trials	Total sugar (g/L)	Initial pH	$FeSO_4$ (g/L)	P_s (mL H_2/L)
1	15	6.0	0.7	3,548.86
2	20	5.5	0.9	4,219.43
3	22.5	5.0	1.2	3,098.43
4	25	4.5	1.5	4,352.86
5	30	4.0	2	3,819.29

3.3.3 Optimization of environmental factors concentrations for hydrogen production from sweet sorghum syrup by mixed cultures

This experiment was designed to optimize the concentrations of three selected variables in order to maximize the hydrogen production and to determine the interactive effects among the tested variables using the Box-Behnken design of RSM.

The three significant independent variables i.e., total sugar, initial pH and $FeSO_4$ from the Plackett-Burman design were selected for design matrix. Hydrogen production ranged from 4,289 to 6,938 mL H_2/L (Table 3). Low hydrogen production, 4,289 mL H_2/L, was obtained in Run 10 while the maximum hydrogen production was obtained in Run 16 (6,938 mL H_2/L).

The multiple regression analysis was applied on the data in Table 3 and the attained second-order polynomial equation could well explain the hydrogen production:

$$Y = 6896.63 - 45.05X_1 + 13.95X_2 + 379.82X_3 - 447.96X_1X_2 + 77.99X_1X_3 + 181.65X_2X_3 - 330.65X_1^2 - 1064.16X_2^2 - 846.47X_3^2 \quad (7)$$

where Y is predicted hydrogen production and X_1, X_2 and X_3 are the coded values of total sugar, initial pH and $FeSO_4$, respectively. A high determination coefficient (R^2 = 0.98) explaining 98% of variability in the response and a high value of the adjusted determination coefficient (adjusted R^2 = 0.95) suggesting a high significance of the model [44]. In addition, a very low probability (P <0.0001) obtained from the regression analysis of variance (ANOVA) demonstrated that the model was significant. A high precision and reliability of the experiments was indicated by a low variation coefficient value of 3.19%.

Only $FeSO_4$ (X_3) had a P -value less than 0.1 (P = 0.0007) (Table 5) indicated a significant effect on hydrogen production. Previous research reported that Fe was the important factor for bio-hydrogen production by *Clostridium*-rich composts [45] as well as mixed anaerobic microflora [46]. This can be explained by the fact that biohydrogen production depends on the hydrogenase activities which are Fe-containing enzymes [47]. *In vivo* activity of hydrogenase in fermentative bacteria was found to decrease with a reduction in Fe [48]. Hydrogen evolves as the final product of reductant disposal from hydrogenase or nitrogenase activity [49] in which the primary electron donor for both enzymes is ferredoxin. Hydrogenase presence in anaerobic bacteria oxidizes reduced ferredoxin to produce molecular hydrogen [50].

Significant interaction effects (P<0.05) were found between total sugar and initial pH (X_1X_2), the square terms of total sugar (X_1^2), initial pH (X_2^2) and $FeSO_4$ (X_3^2) (Table 5). The R^2 value of 0.979 indicated a good agreement between

experimental and predicted values and implied that the mathematical model could predict the hydrogen production very well [35]. These results suggested that total sugar, $FeSO_4$ and initial pH were required for the hydrogen producers to maximize hydrogen production.

In order to obtain the optimal condition for maximizing hydrogen production, Eq. (7) was set to zero with respect to the corresponding variables. It was found that the optimal conditions for the maximal hydrogen production were 25 g/L total sugar, 4.75 initial pH and 1.45 g/L $FeSO_4$ which was found at the moderate condition. At the optimal condition, P_s of 6,897 mL H_2/L was estimated (Table 3). Three-dimensional response surfaces and two-dimensional contour lines based on Eq. (7) were plotted in order to determine the optimum level of each variable and the effects of their interactions on the hydrogen production (Figure 1-3). Figure 1 depicted the response surface plot and corresponding contour curves based on independent variables total sugar (X_1) and initial pH (X_2), while the third independent variable, $FeSO_4$ (X_3), was kept at the optimal level. The two-dimensional contour plot suggested that total sugar and initial pH were interdependent or there was a significant interaction on P_s between total sugar and initial pH (P = 0.0019). A significantly increase on P_s could be achieved when total sugar was increased from 20 to 25 g/L, then slightly decreased when total sugar concentrations were higher than 25 g/L which indicated that substrate inhibition occurred. Figure 1 showed that hydrogen production was increased as initial pH increased from 4.0 to 4.75 and then decreased beyond the level. The pH is one of the most important factors in hydrogen production because it affects metabolic pathways, the duration of lag phase and Fe-hydrogenase activity [48, 47]. A higher initial pH value can delay the beginning of the pH inhibition caused by the metabolic shift from acidogenesis to solventogenesis [51, 52, 53, 54]. Low initial pH value of 4.0-4.5 was reported to cause long lag period [42]. In contrast, high initial pH value, for example 9.0, could decrease lag time but yielded a low hydrogen production [55]. Previous reports indicated that an optimum pH for hydrogen production by anaerobic fermentation was in the range of 5.5–6.0 [56] which is coincided with Kim et al. [57] who reported that acidogenic condition was the most suitable condition to produce hydrogen by anaerobic fermentative consortia.

Figure 2 illustrated the response surface plot and corresponding contour curves based on independent variables total sugar (X_1) and $FeSO_4$ (X_3), while the third independent variable, initial pH (X_2), was kept at the optimal level. The two-dimensional contour plot suggested that total sugar and initial pH was interdependent. The interaction effect between total sugar and $FeSO_4$ (X_1X_3) on hydrogen production was found to be not significant (P = 0.4300) (Table 5). Results indicated that P_s increased when $FeSO_4$ concentration increased from 0.9 g/L to 1.45 g/L and then slightly decreased with a further increase in $FeSO_4$ concentrations.

Figure 3 showed three-dimensional response plots and two-dimensional contour plots showing the effects of $FeSO_4$, initial pH and their mutual interaction on hydrogen production with optimum level of total sugar (25 g/L). The two-dimensional contour plot implied that the interaction effect between initial pH and $FeSO_4$ (X_2X_3) on hydrogen production was not significant with the P-value = 0.0921. Results showed the estimated optimum point occurred at 4.75 of initial pH and 1.45 g/L $FeSO_4$ which confirmed by a shape of circular contour plot.

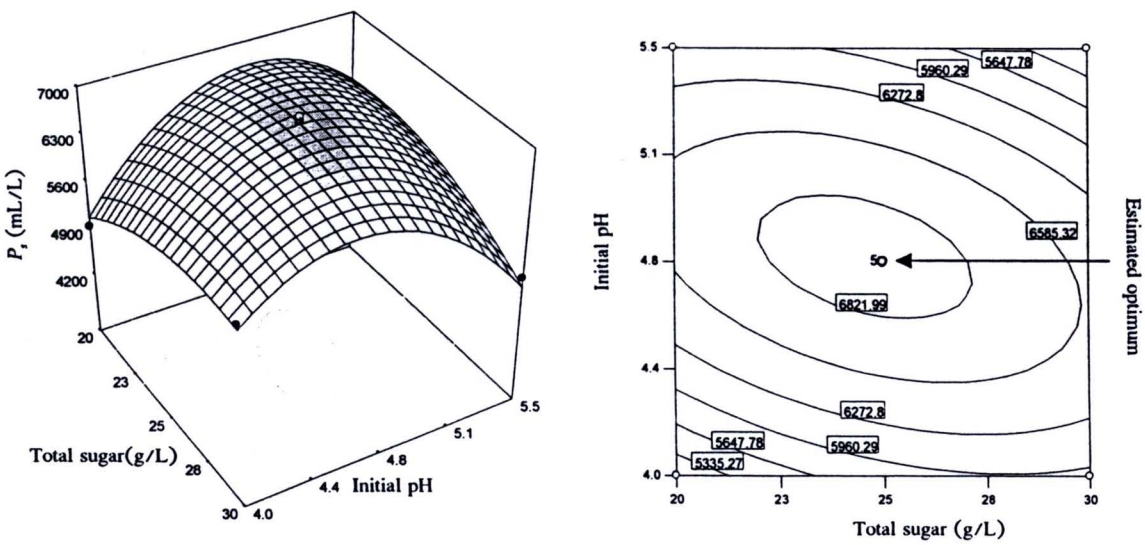

Figure 1 Three-dimensional response plots and two-dimensional contour plots showing the effects of total sugar, initial pH and their mutual interaction on P_s with optimum level of $FeSO_4$ (1.45 g/L).

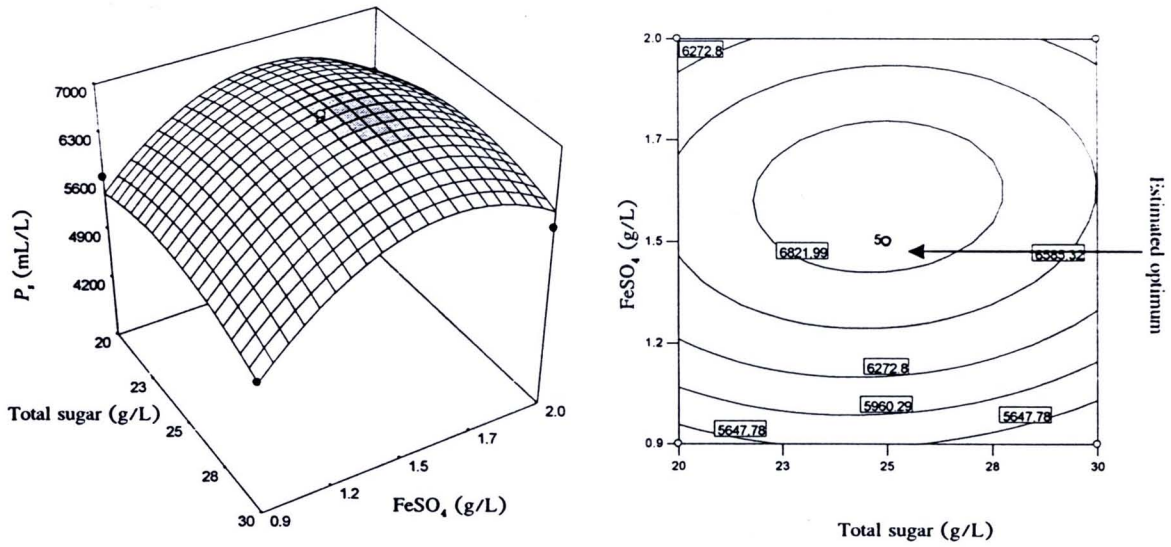

Figure 2 Three-dimensional response plots and two-dimensional contour plots showing the effects of total sugar, $FeSO_4$ and their mutual interaction on P_s with optimum level of initial pH (4.75).

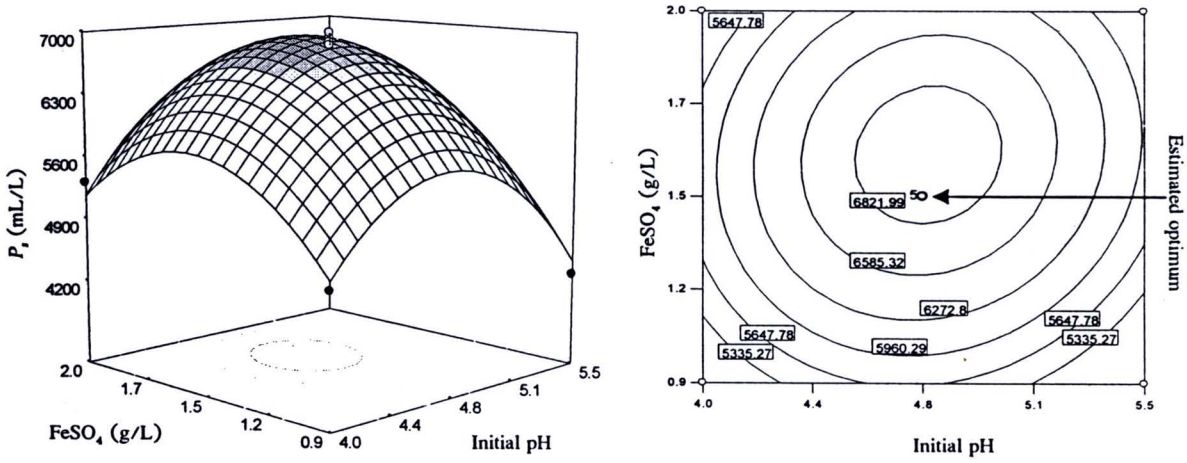

Figure 3 Three-dimensional response plots and two-dimensional contour plots showing the effects of $FeSO_4$, initial pH and their mutual interaction on P_s with optimum level of total sugar (25 g/L).

Table 5 ANOVA and results of regression analysis of Box-Behnken design for hydrogen production.

Term	Sum of squares	Degree of freedom	Coefficient estimate	Standard error	*F*-value	*P*-value *Prob>F*
Intercept	1.115E+007	9	6896.63	83.30	35.70	<0.0001
X_1	16236.02	1	-45.05	65.85	0.47	0.5159
X_2	1555.70	1	13.95	65.85	0.045	0.8383
X_3	1.154E+006	1	379.82	65.85	33.26	0.0007
X_1X_2	8:027E+005	1	-447.96	93.13	23.14	0.0019
X_1X_3	24329.76	1	77.99	93.13	0.70	0.4300
X_2X_3	1.320E+005	1	181.65	93.13	3.80	0.0921
X_1^2	4.603E+005	1	-330.65	90.77	13.27	0.0083
X_2^2	4.768E+006	1	-1064.16	90.77	137.43	<0.0001
X_3^2	3.017E+006	1	-846.47	90.77	86.96	<0.0001
Error	20639.98	4				
Total	1.139E+007	16				

A coefficient of determination (R^2) = 0.979

3.3.4 Confirmation experiment

The confirmation experiment was conducted to verify if the optimum condition obtained from the statistic approach can be practically used to produce maximum hydrogen. Four replicates of batch experiments under optimal condition and control (sweet sorghum syrup) was conducted. Predicted hydrogen production of 6,897 mL H_2/L was obtained at the optimum condition (25 g/L total sugar, initial pH of 4.78 and 1.45 g/L $FeSO_4$) (Table 6). Verification experiment revealed only 0.04% difference between estimated and actual hydrogen production (6,864 mL H_2/L) under the optimal condition and the P_s from the optimum condition was greater than the P_s from the control 287% (Table 6). Results suggested that the optimal conditions attained had the least error and can be practically applied to produce hydrogen from sweet sorghum syrup. The results from the Endo nutrient confirmed the results attained from the Plackett Burman (Table 1) in which the Endo nutrient is not the main effect in the hydrogen production from sweet sorghum syrup by the mixed cultures (Table 6). Table 7 indicated that the predicted maximum hydrogen production was favorable compared with other literature.

Table 6 Experimental design and results for confirmation test.

Run #	Condition	Total sugar (g/L)	Initial pH	$FeSO_4$ (g/L)	P_s (mL H_2/L)	R_m (mL H_2/L/h)	H_2 yield (mol H_2/ mol hexose)
3-17	Optimum	25	4.75	1.45	6896.63	51.66	2.22
8	High	30	4.75	2	6132.27	21.02	1.64
5	Worst	20	4.75	0.9	5462.74	15.36	2.19
-	Endo[a]	20	4.75	-	2464.04	17.26	0.99
-	Control[b]	20	4.75	-	1778.51	12.05	0.71

[a] sweet sorghum syrup contained 0.5 mL/L Endo nutrient

[b] sweet sorghum syrup

Table 7 Comparison of P_s and H_2 yield at the optimum conditions with various substrates and microorganism in batch fermentation

Substrate	Microorganism	Optimum condition	P_s * (mL H_2/L syrup)	H_2 yield (mol H_2/mol substrate)	Ref.
Glucose	*Clostridium* sp. Fanp2	23.75 g/L glucose, 0.159 M phosphate buffer and 13.3 mL/L vitamin solution at 36±1 °C	4,166	2.33 mol H_2/mol hexose	[35]
Sucrose	Mixed anaerobic cultures	24.8 g/L sucrose, pH 5.5 at 34.8°C	2,083	1.92 mol H_2/mol glucose	[34]
Glucose	Heat-treated sludge	10 g/L glucose, pH 5.5 at 41°C	2,935	1.67 mol H_2/mol glucose	[58]
Sucrose	*Clostridium* CGS5	20 g COD sucrose, pH 5.5 at 37°C	1,213	2.78 mol H_2/mol sucrose	[52]
Sweet sorghum syrup	Heat-treated sludge	25 g/L total sugar, initial pH 4.78 and 1.45 g/L $FeSO_4$ at room temperature (30-32 °C)	6,897	2.22 mol H_2/mol hexose	This study

* Specific hydrogen production potential (P_s) was compared with 1 L of medium.

The overall performance of hydrogen production at optimum conditions was shown in Figure 4. Results from the confirmation experiment calculated by the modified Gompertz equation indicated P_s of 6,864 mL H_2/L, R_{max} of 51.66 mL H_2/L/h and 9.26 hours lag time were obtained. Hydrogen production began while VSS decreased after a lag phase which implied that microorganisms did not degrade sugar in sweet sorghum syrup for cell growth instead for hydrogen and metabolites productions (Figure 4a and b). A reduction in VSS might due to a pH drop which was resulted from the accumulated VFAs in the hydrogen fermentation system (Figure 4b). In addition, since the Endo nutrient was not the important parameter in this study (Table 1) therefore a drop of pH to 3.65 was not surprising due to a lack of the buffer in our hydrogen production system. A final pH of 3.65 from an initial pH of 4.78 indicated the accumulation of these acids. Mu et al. [34] and Zhu and Yang [51] described that pH affects the bacterial growth rate and the change of pH could alter the number of microbial population in the system by destroying the cell's ability to maintain internal pH [51].

3.4 Conclusions

Three significant variables affecting hydrogen production from sweet sorghum syrup by anaerobic mixed cultures i.e., total sugar, initial pH and $FeSO_4$ were selected by the Plackett- Burman design experiments. These variables were optimized by the Box-Benhken design to maximize hydrogen production. Optimum conditions attained were 25 g/L total sugar, initial pH of 4.75 and 1.45 g/L $FeSO_4$. At the optimum condition, estimated hydrogen production was 6,864 mL H_2/L which was only 0.04% difference from the actual value, 6,897 mL H_2/L, obtained from the confirmation experiment which suggested that the optimal conditions obtained can be practically applied to produce hydrogen from sweet sorghum syrup with the least error.

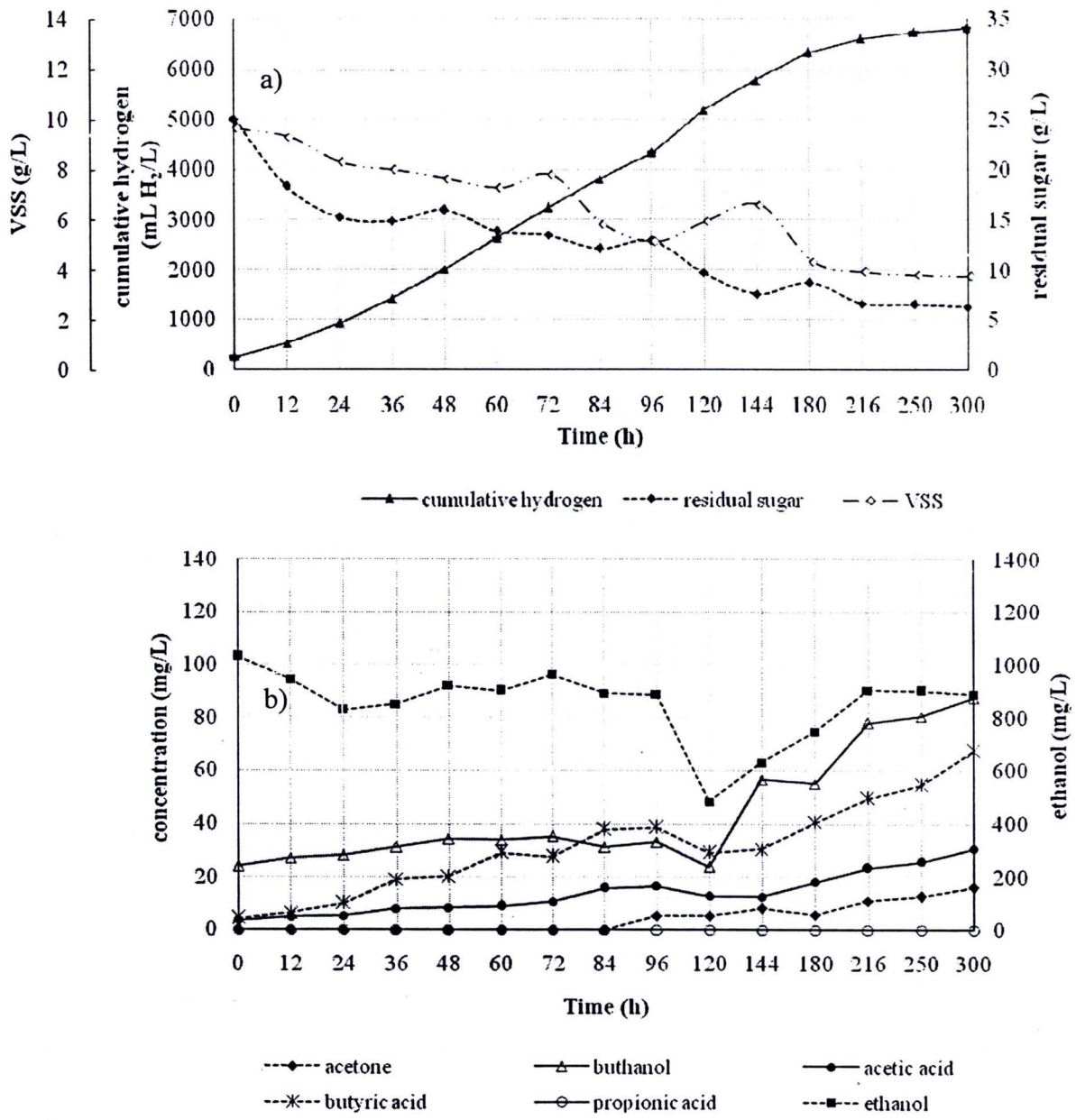

Figure 4 Overall performance of hydrogen production at optimum conditions; (a) cumulative hydrogen, volatile suspended solid (VSS) and residual sugar concentration; (b) metabolites production

3.5 References

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