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Bio-Hydrogen Production from Pineapple Waste Extract by Anaerobic Mixed Cultures

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Abstract: A statistical experimental design was employed to optimize factors that affect the production of hydrogen from the glucose contained in pineapple waste extract by anaerobic mixed cultures. Results from Plackett-Burman design indicated that substrate concentration, initial pH and $FeSO_4$ concentration had a statistically significant ($p \leq 0.05$) influence on the hydrogen production potential (P_s) and the specific hydrogen production rate (SHPR). The path of steepest ascent was undertaken to approach the optimal region of these three significant factors which was then optimized using response surface methodology (RSM) with central composite design (CCD). The presence of a substrate concentration of 25.76 g-total sugar/L, initial pH of 5.56, and $FeSO_4$ concentration of 0.81 g/L gave a maximum predicted P_s of 5489 mL H_2/L, hydrogen yield of 1.83 mol H_2/mol glucose, and SHPR of 77.31 mL H_2/g-volatile suspended solid (VSS) h. A verification experiment indicated highly reproducible results with the observed P_s and SHPR being only 1.13% and 1.14% different from the predicted values.

Keywords: pineapple waste extract; bio-hydrogen

1. Introduction

Hydrogen is a promising alternative energy carrier and is also considered to be a clean energy. It only produces water when combusted with oxygen and has an energy content 2.75 times higher than hydrocarbon fuels [1,2]. Hydrogen can be produced using biological, chemical, and physical processes. The biological hydrogen production process has gained more interest than chemical and physical processes because it is a sustainable process that consumes less energy. Biological hydrogen production can be divided into two types, *i.e.*, a phototrophic process and a dark fermentation process. Dark fermentation has advantages over the phototrophic process in terms of its ability to continuously produce hydrogen from a variety of feedstocks without an external input of energy [3].

Pineapple waste is basically composed of residual pulp, peel, and cores. Pineapple waste is not considered attractive as an animal feed because of its high fiber content, high soluble carbohydrate and low protein content [4]. However, the pineapple waste extract, *i.e.*, the juice obtained after the pineapple waste has been squeezed by a presser, mainly contains sugars and organic acids that can be utilized as the substrates in the production of hydrogen and ethanol [5–8]. Due to its composition, we attempted to use the pineapple waste extract in hydrogen production using anaerobic mixed cultures. In order to achieve maximum hydrogen production, there was a need to provide suitable fermentation conditions, especially the environmental factors such as temperature, pH, nutrient addition, buffer, substrate concentration and ferrous iron. The appropriate concentration of substrate could enhance bacterial growth and activity [9]. An excessive substrate concentration can cause a build-up of volatile fatty acid (VFAs) in the fermentation broth, leading to a low pH in the system. Moreover, pineapple waste extract contains a high amount of short chain organic acids, which in turn means that the extract would have a low pH and a high acid concentration. A high acidic content in the substrate can cause adverse effects on hydrogen production. Thus, the addition of a buffer to counteract a decrease in pH is needed. Endo–nutrient and iron are often used to enrich microorganisms capable of producing hydrogen. The important elements contained in the Endo–nutrient such as Cu^{2+}, Co^{2+}, Mg^{2+} are essential for microorganism growth and activity. Iron is the important element for the activity of hydrogenase, which is an important enzyme for hydrogen production [10,11]. Initial pH is a physical factor that also has a great influence on hydrogen production. In general, the appropriate range of initial pH for hydrogen production and cell growth is in the ranges of 4.0–7.0 [10,11]. If the initial pH is outside the optimal range, and if enough buffer capacity is not present, the hydrogen production process will be inhibited [10,11].

Accordingly, the goal of this research was to optimize the environmental key factors that influence the biohydrogen production from pineapple waste extract by anaerobic mixed cultures. A Plackett-Burmann design was used to screen the significant variables. Response surface methodology (RSM) with central composite design (CCD) was used to optimize the levels of these variables. The results were expected to provide the optimal conditions for improving and enhancing the hydrogen production potential from pineapple waste extract.

2. Results and Discussion

2.1. Screening of the Significant Variables Affecting Hydrogen Production from Pineapple Waste Extract by Anaerobic Mixed Cultures

The key factors that affected hydrogen production from pineapple waste extract were screened using the Plackett-Burman design. Table 1 shows the significance and effects of each variable on hydrogen production potential (P_s) and specific hydrogen production rate (SHPR). The effect of each variable was calculated by the difference between the averages of measurements made at the high and low level of the factor.

The sign of the effect value (Ex_i) showed the influence of the variable on P_s and SHPR. The negative sign of the effect showed that the influence of a variable on the response should be lower than that of the low level (-1), whereas the positive sign of the effect showed that the influence of the variable on response should be higher than that of the high level ($+1$). The results indicated that the influence of $FeSO_4$ concentration, initial pH, and $NaHCO_3$ concentration were greater at a low level while the influence of Endo–nutrient and substrate concentration were greater at a high level (Table 1). The significance of each variable was determined by the probability value. Results indicated that $FeSO_4$ concentration, initial pH, and substrate concentration had a significant effect on P_s and SHPR ($p \leq 0.05$). Therefore, these three variables were chosen for the next optimization step.

Table 1. Levels of the variables and statistical analysis in Plackett–Burman design for P_s and SHPR.

Code	Variable	Low level	High level	P_s (mL H$_2$/L)			SHPR [mL H$_2$/g-volatile suspended solid (VSS) h]		
				Effect (Ex_i)	F-Values	p-Value Prob > F	Effect (Ex_i)	F-Values	p-Value Prob > F
X_1	FeSO$_4$ concentration (g/L)	1	2	−549.00	13.38	0.0106	−7.73	13.38	0.0018
X_2	Initial pH	5	7	−1189.19	62.77	0.0002	−16.74	62.77	0.0002
X_3	Endo–nutrient (mL/L)	0.5	2	2.36	0.002	0.9879	0.03	0.002	0.9879
X_4	Substrate concentration (g-total sugar/L)	10	20	421.1	7.87	0.0309	5.93	7.87	0.0309
X_5	NaHCO$_3$ concentration (g/L)	1	5	−97.01	0.42	0.542	−1.37	0.42	0.542

2.2. The Path of Steepest Ascent

The path of steepest ascent was used in order to approach the proper direction of the changing variable. Results indicated that an increase in substrate concentration from 20 to 25 g-total sugar/L resulted in an increase in P_s and SHPR by 22.74% and 22.71% (Table 2), respectively. An increase in substrate concentration greater than 25 g-total sugar/L caused a decrease in P_s and SHPR by 13.18% and 13.16%, respectively. Based on these results, the selected range of substrate concentration of 20–30 g-total sugar/L was used in the optimization step through RSM with CCD.

P_s and SHPR were increased with a decrease in initial pH from 6.0 to 5.5. A further decrease in the initial pH to less than 5.5 resulted in a decrease in P_s and SHPR (Table 2). The highest P_s and SHPR of 4680 mL H$_2$/L and 65.87 mL H$_2$/g-VSS h, respectively, were achieved at the initial pH of 5.5.

Therefore the selected range of initial pH used in the optimization experiment was chosen to be between 6.0 and 5.0 g/L.

The effect of $FeSO_4$ concentration on P_s and SHPR was also investigated. The highest P_s and SHPR of 4680 mL H_2/L and 65.87 mL H_2/g-VSS h were obtained at 0.8 g/L $FeSO_4$. Based on this result, the $FeSO_4$ concentration that ranged between 1.0 to 0.6 g/L was used in the optimization experiment. The results from the path of steepest ascent indicated that the selected ranges of substrate concentration, initial pH and $FeSO_4$ concentration to be used in the optimization study were 20–30 g-total sugar/L, 6.0–5.0 g/L, and 1.0–0.6 g/L, respectively.

Table 2. Results and experimental design of the path of steepest ascent.

Run	Substrate concentration (g-total sugar/L)	Initial pH	$FeSO_4$ concentration (g/L)	P_s (mL H_2/L)	SHPR (mL H_2/g-VSS h)
1	20	6.0	1.0	3616	50.91
2	25	5.5	0.8	4680	65.87
3	30	5.0	0.6	4063	57.20
4	35	4.5	0.4	3033	42.70
5	40	4.0	0.2	2702	38.03

2.3. Optimization of Substrate Concentration, Initial pH, and $FeSO_4$ Concentration on P_s Using RSM with CCD

CCD was used to optimize substrate concentration, initial pH, and $FeSO_4$ concentration on P_s. Regression analysis of the data from Table 3 resulted in the quadratic equation [Equation (1)] as follows:

$$Ps = +5317.17 + 145.00X_1 + 103.31X_2 + 79.71X_3 - 94.42X_1X_2 - 119.46X_1X_3$$
$$+93.00X_2X_3 - 413.53X_1^2 - 430.37X_2^2 - 541.86X_3^2 \tag{1}$$

where P_s is the hydrogen production potential; and X_1, X_2 and X_3 are the substrate concentration, initial pH, and $FeSO_4$ concentration, respectively. The model showed a high determination coefficient ($R^2 = 0.99$), explaining 99% of the variability in the response. A very low probability ($p < 0.0001$) obtained from the regression analysis of variance (ANOVA) demonstrated that the model was significant (Table 4).

The significance of the variables was determined by the probability values (Table 4). Results showed that substrate concentration (X_1), initial pH (X_2), and $FeSO_4$ concentration (X_3) had a significant individual effect on P_s ($p \leq 0.05$). The quadratic model terms of all variables (X_1^2, X_2^2 and X_3^2) are highly significant ($p \leq 0.0001$). A significant interaction effect on P_s was found between substrate concentration and initial pH (X_1X_2), substrate concentration and $FeSO_4$ concentration (X_1X_3), and initial pH and $FeSO_4$ concentration (X_2X_3) ($p \leq 0.05$). Based on the regression analysis of the model [Equation (1)], the maximum P_s of 5425 mL H_2/L could be predicted at the substrate concentration of 25 g-total sugar/L, initial pH of 5.56 and $FeSO_4$ concentration of 0.78 g/L. The response surface plots based on Equation (1), with one variable kept constant at their optimum values and variations of the other two variables within the experimental range are depicted in Figure 1. Results indicated that the optimum conditions for maximum P_s fell well inside the design boundaries.

Table 3. Central composite experimental design matrix defining pH, substrate concentration and FeSO$_4$ concentration for optimizing the fermentative hydrogen production process and the corresponding experimental results.

Run	Substrate concentration (X_1) (g-total sugar/L)		Initial pH (X_2)		FeSO$_4$ concentration (X_3) (g/L)		P_s (mL H$_2$/L)		SHPR (mL H$_2$/g-VSS h)	
	Code value	Actual value	Code value	Actual value	Code value	Actual value	Observed	Predicted	Observed	Predicted
1	0.00	25.00	0.00	5.50	0.00	0.80	5332	5317	75.05	74.85
2	0.00	25.00	0.00	5.50	0.00	0.80	5306	5317	74.68	74.85
3	1.00	30.00	1.00	6.00	1.00	1.00	4137	4139	58.23	58.21
4	−1.00	20.00	−1.00	5.00	1.00	1.00	3875	3695	54.54	52.27
5	0.00	25.00	0.00	5.50	0.00	0.80	5337	5317	75.13	74.85
6	1.00	30.00	−1.00	5.00	−1.00	0.60	4237	4200	59.64	59.07
7	1.68	33.41	0.00	5.50	0.00	0.80	4464	4391	62.84	61.79
8	−1.00	20.00	−1.00	5.00	−1.00	0.60	3562	3483	50.14	49.03
9	0.00	25.00	0.00	5.50	−1.68	0.46	3606	3651	50.76	51.36
10	0.00	25.00	−1.68	4.66	0.00	0.80	3815	3926	53.7	55.33
11	0.00	25.00	0.00	5.50	1.68	1.14	3853	3919	54.24	55.24
12	1.00	30.00	1.00	6.00	−1.00	0.60	3957	4032	55.7	56.85
13	−1.00	20.00	1.00	6.00	1.00	1.00	4317	4276	60.77	60.21
14	1.00	30.00	−1.00	5.00	1.00	1.00	3916	3935	55.12	55.39
15	0.00	25.00	0.00	5.50	0.00	0.80	5194	5317	73.12	74.85
16	−1.68	16.59	0.00	5.50	0.00	0.80	3721	3904	52.38	55.04
17	0.00	25.00	0.00	5.50	0.00	0.80	5340	5317	75.17	74.85
18	−1.00	20.00	1.00	6.00	−1.00	0.60	3789	3692	53.33	51.93
19	0.00	25.00	1.68	6.34	0.00	0.80	4275	4274	60.18	60.14
20	0.00	25.00	0.00	5.50	0.00	0.80	5413	5317	76.2	74.85

Table 4. Model coefficients estimated by multiple linear regressions.

Factor	P_s (mL H$_2$/L)		SHPR (mL H$_2$/g-VSS h)	
	Coefficient estimate	Probability	Coefficient estimate	Probability
Model	5317.17	<0.0001	74.85	<0.0001
X_1	145	0.0009	2.01	0.0012
X_2	103.31	0.0076	1.43	0.0101
X_3	79.71	0.0278	1.15	0.0286
X_1X_2	−94.42	0.0419	−1.28	0.0543
X_1X_3	−119.46	0.0145	−1.73	0.0148
X_2X_3	93	0.0445	1.26	0.0576
X_1^2	−413.53	<0.0001	−5.81	<0.0001
X_2^2	−430.37	<0.0001	−6.05	<0.0001
X_3^2	−541.86	<0.0001	−7.62	<0.0001

Figure 1. Response surface plots showing (**a**) the effects of initial pH, substrate concentration, and their mutual interaction on P_s with optimum level of FeSO$_4$ concentration (0.78 g/L); (**b**) the effects of FeSO$_4$ concentration, substrate concentration and their mutual interaction on P_s with optimum level of initial pH (5.56); and (**c**) the effects of FeSO$_4$ concentration, initial pH and their mutual interaction on P_s with optimum level of substrate concentration (25 g-total sugar/L).
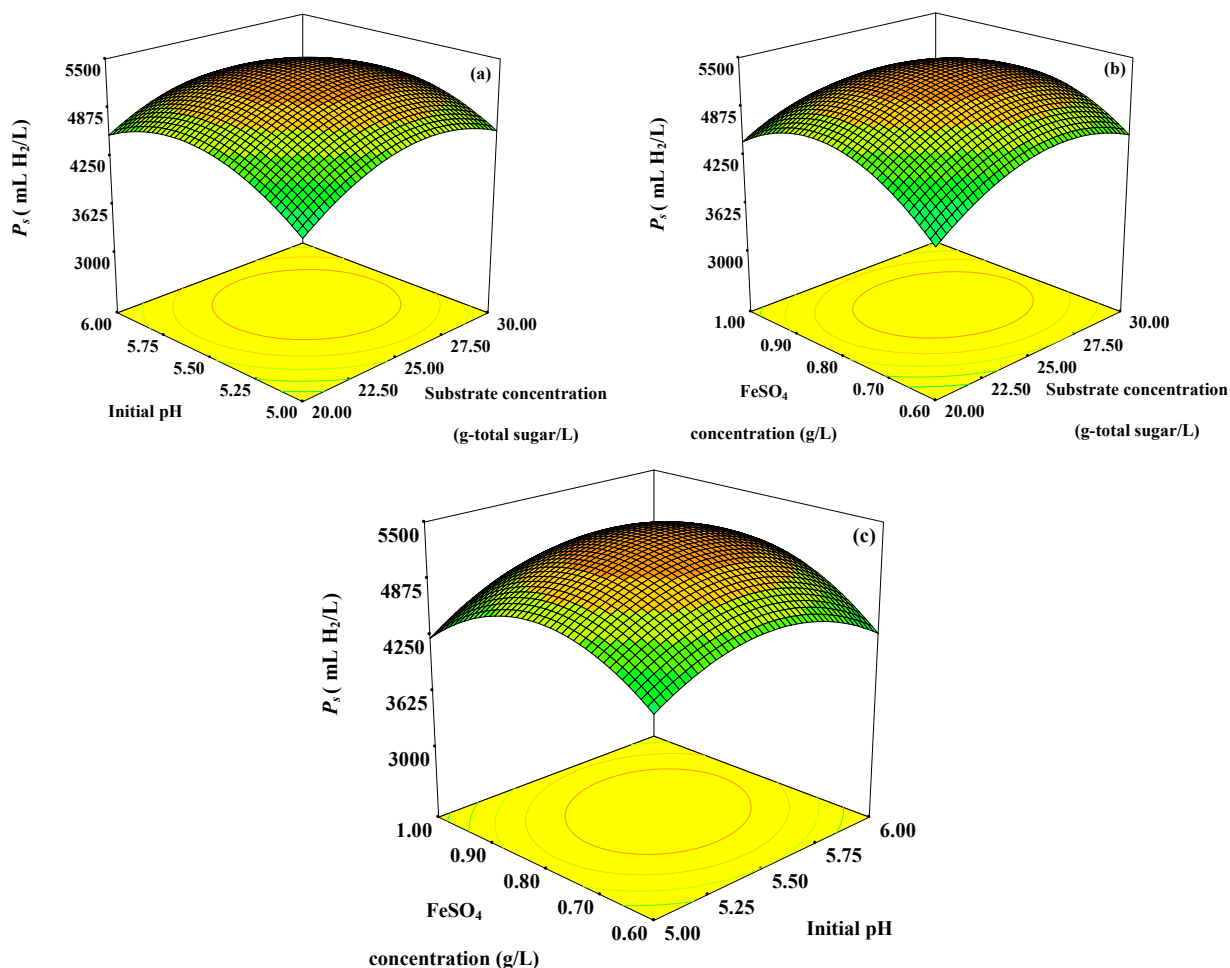
The P_s increased with increasing substrate concentration up to 25 g-total sugar/L. Further increase in substrate concentration greater than 25 g-total sugar/L resulted in lower P_s (Figure 1a,b). This might be due to substrate and product (VFAs) inhibitions. A high substrate concentration, not exceeding the substrate inhibition level, in the fermentation broth resulted in a high P_s because substrate was used to produce hydrogen. An increase in the substrate concentration higher than optimum level might develop osmotic pressure inside the microbial cells, which can cause cell damage due to the permeation of water molecules out of the cells [12]; hence a reduction of hydrogen production occurred. In addition, an increase in substrate concentration could have led to a partial pressure of hydrogen build-up in the fermentation system. When the partial pressure accumulates in the headspace of the serum bottle to a certain level, hydrogen production will be switched to solvent production, thus hydrogen production was inhibited [13]. Therefore, the substrate concentration should be optimized in order to prevent the shock loads of the system [14].

The P_s increased when the initial pH increased from pH 5.00 to 5.56. However, a further increase in initial pH to levels greater than pH 5.56 resulted in a decrease in P_s (Figure 1a,c). The initial pH is an important factor in hydrogen production because it affects metabolic pathways, iron (Fe)-hydrogenase activity and the duration of the lag phase [13–18]. When the initial pH was high, the metabolic pathway will have shifted from acidogenesis to solventogenesis [13–18]. The low initial pH value of pH 4.0–4.5 was reported to cause a long lag period [13]. In contrast, a high initial pH value, for example pH 9.0 could decrease the lag time but it would result in a low level of hydrogen production [13–19].

An increase in the $FeSO_4$ concentration from 0.60 to 0.78 g/L increased the P_s (Figure 1a,c). However, the results indicated that P_s was decreased when the $FeSO_4$ concentration was greater than 0.78 g/L. Iron is needed in hydrogen production by anaerobic fermentation because it forms ferredoxin and hydrogenase, which is directly related to the hydrogen production process. Iron can affect the fermentative production of hydrogen by influencing the activity of hydrogenases [20–23]. The optimum iron concentration could enhance fermentative hydrogen production by mixed cultures, while lower or higher iron concentrations than optimum range could reduce the activity of hydrogenases [20–23].

2.4. Optimization of Substrate Concentration, Initial pH, and FeSO₄ Concentration on SHPR Using RSM with CCD

Optimization of substrate concentration, initial pH, and $FeSO_4$ concentration on SHPR was conducted using RSM with CCD. The observed and predicted values of SHPR are presented in Table 3. Multiple regression analysis was applied to the data in Table 3 and the second-order polynomial equation [Equation (2)] obtained could well explain the SHPR values:

$$SHPR = +74.85 + 2.01X_1 + 1.43X_2 + 1.15X_3 - 1.28X_1X_2 - 1.73X_1X_3$$
$$-1.26X_2X_3 - 5.81X_1^2 - 6.05X_2^2 - 7.62X_3^2 \tag{2}$$

where SHPR is the specific hydrogen production rate and X_1, X_2 and X_3 are the codes of substrate concentration, initial pH, and $FeSO_4$ concentration, respectively. The quadratic regression model indicated that the model was significant with a low probability ($p < 0.0001$). All variables, *i.e.*, substrate concentration, initial pH, and $FeSO_4$ concentration showed probability values of less than 0.05, which indicated their significant individual effect on SHPR (Table 4). The quadratic model terms of all variables (X_1^2, X_2^2 and X_3^2) are highly significant ($p \leq 0.0001$). A significant interactive effect on SHPR was found between substrate concentration and $FeSO_4$ concentration (X_1X_3) (Table 4). Based on the regression analysis of the model [Equation (2)], the maximum SHPR of 75.27 mL H_2/g-VSS h could be predicted at the substrate concentration of 25.06 g-total sugar/L, initial pH of 5.50 and $FeSO_4$ concentration of 0.81 g/L.

The response surface plots based on Equation (2), with one variable kept constant at their optimum values and variations of the other two variables within the experimental range, are depicted in Figure 2. SHPR increased when the substrate concentration increased from 20 to 25 g-total sugar/L and then SHPR slightly decreased with a further increase in substrate concentration (Figure 2a,b). The substrate limitation and substrate inhibition effects might be responsible for our findings [19,24,25]. Within the appropriate range, an increase in substrate concentration could improve the ability of hydrogen producing bacteria to produce hydrogen during the fermentation process [19,24,25]. However, a high

substrate concentration could result in a high concentration of the short chain organic acid contained in pineapple waste extract and a low pH of the fermentation broth, which might be toxic to the growth of hydrogen producing bacteria.

Figure 2. Response surface plots showing: (**a**) the effects of initial pH, substrate concentration, and their mutual interaction on SHPR with optimum level of FeSO$_4$ concentration (0.81 g/L); (**b**) the effects of FeSO$_4$ concentration, substrate concentration and their mutual interaction on SHPR with optimum level of initial pH (5.50); and (**c**) the effects of FeSO$_4$ concentration, initial pH and their mutual interaction on SHPR with optimum level of substrate concentration (25.06 g-total sugar/L).
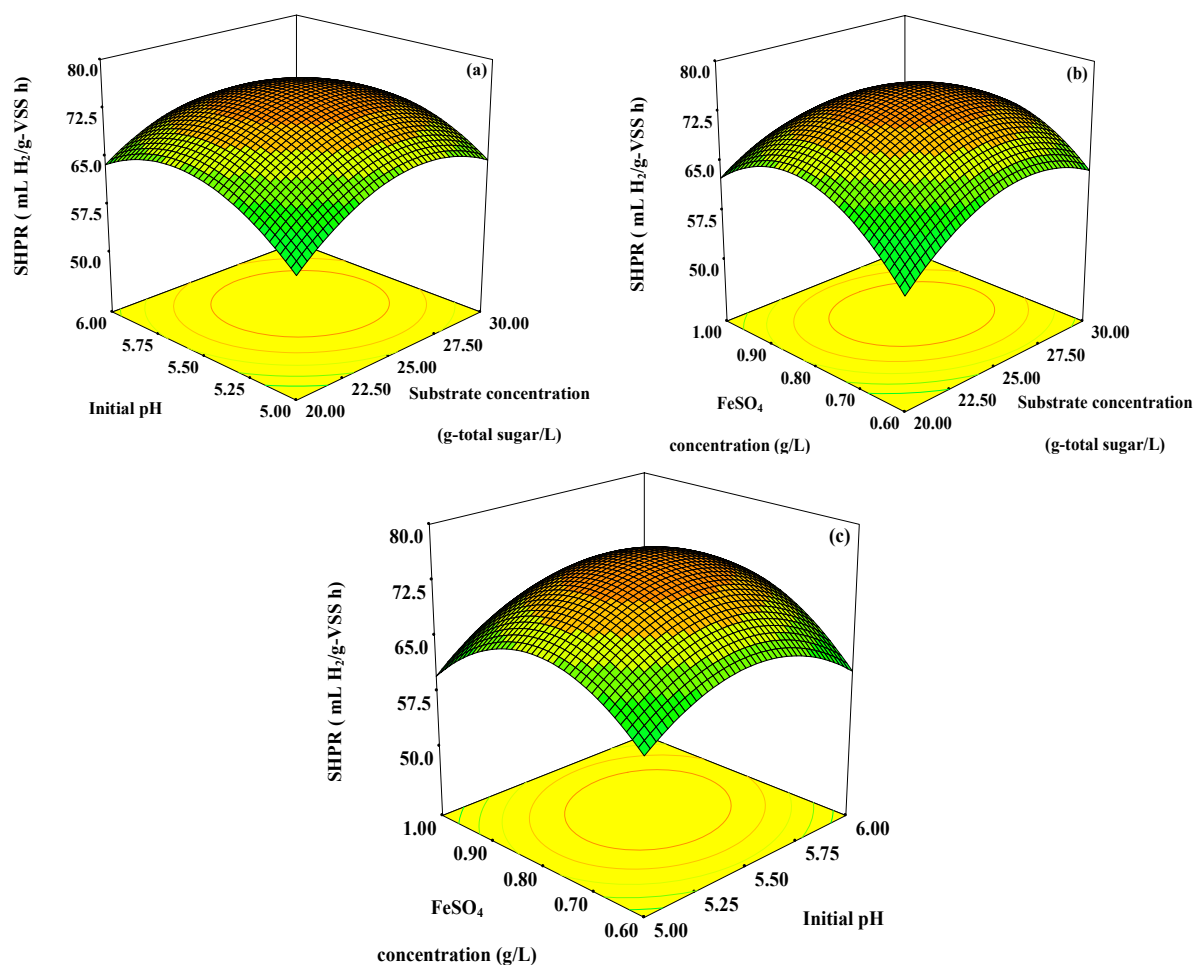
SHPR increased with an increase in initial pH from 5.00 to 5.50 and decreased with an increase in initial pH greater than 5.50 (Figure 2a,c). Thus, a high initial pH causes low SHPR due to the activity of hydrogenase enzyme being inhibited at a strong basidic condition [15,17]. Our results showed that a suitable initial pH is important to maximize hydrogen production from pineapple waste extract. However, the appropriate range of initial pH for hydrogen production and microbial growth is varied according to the cultivation conditions, such as the inocula used, substrate concentration, and incubation temperature [18,19].

SHPR rapidly increased when the $FeSO_4$ concentration was increased up to 0.81 g/L, then the SHPR gradually decreased when the $FeSO_4$ concentration was higher than 0.81 g/L (Figure 2b,c). Iron is an important factor affecting hydrogen production as previously described in the introduction. Thus in the optimal range, an increase in iron concentration not exceeding the optimum level could enhance the SHPR from pineapple waste extract.

2.5. Confirmation Experiment

The analysis of the P_s and SHPR [Equations (1) and (2)] model suggested that in order to obtain the maximum P_s and SHPR, substrate concentration, initial pH, and $FeSO_4$ concentration should be optimized at 25.76 g-total sugar/L, 5.56 and 0.81 g/L, respectively (Table 5). A P_s and SHPR of 5489 mL H_2/L and 77.31 mL H_2/g-VSS h were predicted under the optimum conditions.

In order to confirm the validity of the statistics based experimental strategy, three replicates of each batch experiment were performed under the optimum conditions. The P_s and SHPR results indicated that the observed P_s and SHPR (5427 mL H_2/L and 76.43 mL H_2/g-VSS h) are in close agreement with the predicted values of P_s and SHPR with only 1.13% and 1.14% differences. Results suggested that the model obtained from the CCD experiment is valid.

**Table 5.** Results and experimental design of confirmation test.

Condition	Substrate concentration (g-total sugar /L)	Initial pH	$FeSO_4$ concentration (g/L)	P_s (mL H_2/L)	Hydrogen yield (HY) (mol H_2/mol glucose)	SHPR (mL H_2/g-VSS h)
Optimum	25.76	5.56	0.81	5489	1.83	77.31
High	30	6	1	4172	1.30	58.73
Worst	20	4	0.6	3442	1.71	48.45
Endo [a]	20	5.56	-	2014	1.08	28.35
Control [b]	20	5.56	-	1874	1.04	26.37

Notes: [a] Pineapple waste extract contained 0.5 mL/L Endo−nutrient; [b] Pineapple waste extract.

The biogas produced at the optimum conditions contained hydrogen (28%) and carbon dioxide (72%). No methane gas was detected. The time course of production of P_s and SHPR obtained at the optimum conditions is depicted in Figure 3a. As shown in Figure 3a, after 4 h of lag phase, the P_s and SHPR increased greatly. During fermentation time, P_s increased over time and remained constant at the maximum level of 5427 mL H_2/L. SHPR increased and reached a maximum level of 76.43 mL H_2/g-VSS h then decreased after 30 h (Figure 3a). Figure 3b illustrates the formation of VFAs and alcohols accompanying the production of hydrogen obtained at the optimum conditions. After a lag phase, the concentrations of VFAs and alcohols sharply increased. The fermentation products were butyric acid, acetic acid, ethanol and propionic acid. The detection of butyric and acetic acids was a good indicator that efficient hydrogen production had been achieved [25] and that the fermentation type was butyrate-acetate type fermentation.

The confirmation results in the treatment with Endo−nutrient addition and control (Table 5) confirmed the results attained from the Placktett-Burman (Table 1) that the Endo−nutrient does not have an individual effect on hydrogen production from pineapple waste extract by anaerobic mixed cultures.

Figure 3. (**a**) P_s and SHPR in the confirmation experiment at optimum condition; and (**b**) development of soluble metabolite products at the optimum condition.

(**a**)   (**b**)
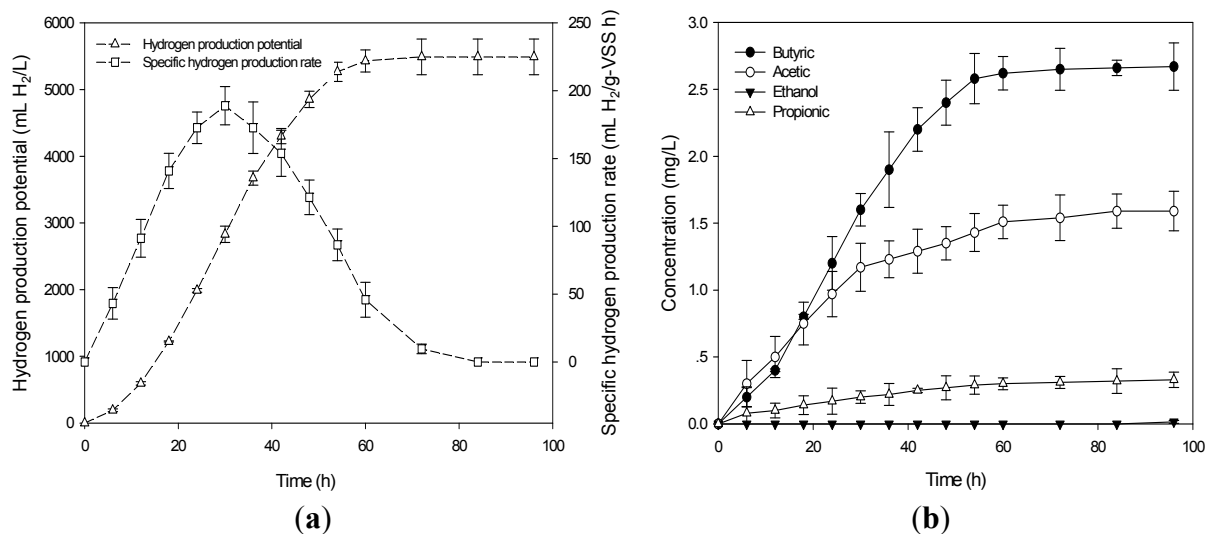
The maximum hydrogen yield (HY) (1.82 mol H_2/mol glucose) obtained in this study was favorable compared with other results reported in the literature search (Table 6). However, the P_s and HY of Saraphirom and Reungsang [26] were 1.26 and 1.21 times than ours, respectively, while the HY of Pan *et al.* [27] was 1.27 times higher than our HY. This is not surprising since the types/sources of inoculums and substrates used to produce hydrogen in their studies were different to those in our study. In addition, the P_s, HY, and SHPR obtained from optimum conditions were approximately 2.93, 1.75, and 2.93, respectively, times higher than control (*i.e.*, 20 g/L pineapple waste extract without an addition of ferrous iron and Endo−nutrient) indicating a significant enhancement of P_s, HY, and SHPR by adding ferrous iron.

Table 6. Comparison of P_s and HY at the optimum conditions with various inoculums and substrates in the experimental batch.

Inoculums	Substrate	Optimum conditions	P_s (mL H_2/L)	HY (mol H_2/mol glucose)	References
Anaerobic sludge	glucose	26.80 g/L glucose, pH 7.0 and 39.3 °C	3999	1.24	[28]
Anaerobic sludge	glucose	9.70 g/L glucose, pH 5.7 and 38 °C	-	1.75	[29]
Clostridium sp. Fanp2	glucose	23.75 g/L glucose, 0.159 M phosphate buffer and 13.3 mL/L vitamine solution	4166	2.33	[27]
Heat-treated sludge	glucose	9.70 g/L glucose, pH 5.5 and 41 °C	2935	1.67	[30]
Heat-treated sludge	Sweet sorghum syrup	25 g/L glucose, pH 4.78 and 1.45 g/L $FeSO_4$	6897	2.22	[26]
Heat-treated sludge	Pineapple waste extract	25.76 g/L glucose, pH 5.56, 0.81 g/L $FeSO_4$ and 35 °C	5489	1.83	This study

3. Experimental Section

3.1. Preparation of Feedstock

Pineapple waste (residual peel and core waste) was obtained from fruit shops at Khon Kaen University, Khon Kaen, Thailand. The pineapple waste was chopped into small pieces using knives before being crushed in a blender. The crushed pineapple waste was squeezed by a presser to extract the juice before being filtered through a thin cloth and then concentrated by heating. The sugar concentration of the pineapple extract was 351 g-total sugar/L. Total nitrogen and total phosphorus of the extract were 0.50 and 0.30 g/L, respectively. The initial pH of the extract was 4.10. The extract was kept in the freezer at −20 °C until used. It was thawed in a refrigerator at 4 °C and centrifuged at 6000 rpm for 10 min to separate the solids prior to being used to produce hydrogen.

3.2. Inoculums Preparation

Anaerobic seed sludge was obtained from a full-scale anaerobic digester of the upflow anaerobic sludge blanket (UASB) reactor of a brewery company, Khon Kaen, Thailand. The UASB is used to produce methane from the wastewater of the beer production process. The anaerobic seed sludge was pre-heated at 105 °C for 3 h in a drying oven (LDO-100E, Daihan Labtech Co., Ltd., Namyangju, Korea) in order to deactivate hydrogen consumers. The pH level and biomass concentration in terms of VSS of the sludge were 6.8 and 7.4 g-VSS/L, respectively. In order to prepare the inocula, pre-heated sludge was cultivated in 20 g-total sugar/L of pineapple waste extract and supplemented with 0.5 mL/L of Endo−nutrient solution [31]. The culture was incubated in a water bath shaker at 150 rpm for 36 h, before being used as the inocula in the batch experiment.

3.3. Optimization Procedure

3.3.1. Placket-Burman Design

A Placket-Burman design was used to screen the significant variables that influence the production of hydrogen from pineapple waste extract by anaerobic mixed cultures. The investigated experimental parameters were substrate concentration, initial pH, $FeSO_4$ concentration, Endo–nutrient addition, and $NaHCO_3$ concentration. The experimental design [32] was based on the first-order model:

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

where Y_i are the responses *i.e.*, P_s and SHPR, β_0 is the model intercept; β_i is the linear coefficient; and X_i is the level of the independent variable. Each of variables was examined in two levels: −1 for low level and +1 for high level. The factors significant at the 95% level ($p \leq 0.05$) were considered to have a significant effect on hydrogen production and were then used in the optimization step. The level of each factor and experimental design matrix used in the experimental design are shown in Tables 1 and 7. The effect of each variable was determined by the following equation:

$$E_{xi} = 2(\sum M_{i+} - M_{i-})/N \tag{4}$$

where, Ex_i is the concentration effect of the tested variable, M_{i+} and M_{i-} are P_s and SHPR from runs where the variable (X_i) measured was present at high and low concentrations, respectively, and N is the number of runs (13). In order to approach the area of the optimum level, the next experiment was carried out along the path of steepest ascent (Table 2). The direction of the maximum increase in P_s and SHPR was yielded by the gradient of the regressed polynomial. The factors significant at the 95% level ($p \leq 0.05$) were considered to have a significant effect on the response and were thus used for further optimization by the steepest ascent.

Table 7. Plackett–Burman design matrix for evaluating factors influencing P_s and SHPR from pineapple waste extract by anaerobic mixed cultures.

Run	FeSO$_4$ concentration (g/L)		Initial pH		Endo−nutrient addition (mL/L)		Substrate concentration (g-total sugar/L)		NaHCO$_3$ concentration (g/L)		P_s (mL H$_2$/L)		SHPR (mL H$_2$/g-VSS h)	
	Code value	Actual value	Code value	Actual value	Code value	Actual value	Code value	Actual value	Code value	Actual value	Observed	Predicted	Observed	Predicted
1	−1.00	1.00	−1.00	5.00	−1.00	0.50	1.00	20.00	−1.00	1.00	3220	3250	45.33	44.42
2	−1.00	1.00	−1.00	5.00	1.00	2.00	−1.00	10.00	1.00	5.00	2656	2735	37.39	35.1
3	1.00	2.00	1.00	7.00	−1.00	0.50	−1.00	10.00	−1.00	1.00	1325	1091	18.65	19.92
4	1.00	2.00	−1.00	5.00	−1.00	0.50	−1.00	10.00	1.00	5.00	2399	2183	33.77	29.89
5	0.00	1.50	0.00	4.50	0.00	1.75	0.00	15.00	0.00	4.50	2772	2123	39.02	39.83
6	−1.00	1.00	1.00	7.00	1.00	2.00	1.00	20.00	−1.00	1.00	2207	2064	31.07	29.05
7	−1.00	1.00	1.00	7.00	1.00	2.00	−1.00	10.00	1.00	5.00	1522	1545	21.43	21.75
8	−1.00	1.00	−1.00	5.00	−1.00	0.50	−1.00	10.00	−1.00	1.00	2623	2829	36.92	32.13
9	1.00	2.00	−1.00	5.00	1.00	2.00	1.00	20.00	−1.00	1.00	2704	2704	38.07	36.66
10	1.00	2.00	1.00	7.00	−1.00	0.50	1.00	20.00	1.00	5.00	1008	1415	14.19	15.36
11	1.00	2.00	−1.00	5.00	1.00	2.00	1.00	20.00	1.00	5.00	2705	2607	38.08	38.46
12	1.00	2.00	1.00	7.00	1.00	2.00	−1.00	10.00	−1.00	1.00	952	1093	13.4	14.03
13	−1.00	1.00	1.00	7.00	−1.00	0.50	1.00	20.00	1.00	5.00	2159	1964	30.39	29.01

3.3.2. CCD

CCD was used to optimize the important factors that enhanced P_s and SHPR. A total of 20 runs (Table 3), in triplicate, were conducted. The P_s and SHPR were selected as the dependent output variables. For statistical calculations, the test factors (X_i) were coded as x_i according to the following transformation equation [Equation (5)]:

$$x_i = (X_i - X_0)/\Delta X_i \tag{5}$$

where x_i is the coded value of the variable X_i; X_i is the actual value of the independent variable; X_0 is the actual value of X_i at the center point and X_i is the step change value. A quadratic model [Equation (6)] [33] was used to evaluate the optimization of the key factors:

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

where Y are the predicted responses (P_s and SHPR); x_i is the parameter; β_0 is a constant; β_i is the linear coefficient; β_{ii} is the squared coefficient and β_{ij} is the interaction coefficient. The response variables

are P_s and SHPR. These responses were fitted using a predictive polynomial quadratic Equation (6) in order to correlate the response variable with the independent variables [33]. P_s was calculated by dividing the amount of hydrogen production (mL H_2) by the working volume of the medium (L). SHPR was calculated by dividing the hydrogen production rate (mL H_2/h) by the amount of biomass concentration in terms of VSS (g-VSS). HY (mol H_2/mol hexose) was calculated by dividing the molar amount of hydrogen production (mol H_2/L) by the substrate consumption (mol hexose/L). The statistical software Design-Expert (Demo version 7.0, Stat-Ease, Inc., Minneapolis, MN, USA) was used for design, modeling and plotting the graphical analysis of the experimental data. The conditions of each trial are shown in Table 3.

3.4. Biohydrogen Production

The biohydrogen production experiment was conducted in 120 mL serum bottles with a working volume of 70 mL. The fermentation broth contained different levels and concentrations of parameters, according to the design (Tables 2, 3, and 7). The serum bottles were capped with rubber stoppers and flushed with nitrogen gas in order to create anaerobic conditions. The bottles were incubated at 35 °C, and 150 rpm in a shaking water bath. At designated times the gas volume was measured by releasing the pressure in the bottles using a wetted glass syringe [34]. The effluent was collected by using a glass syringe and analyzed for VFAs and alcohol by gas chromatography (GC) equipped with a flame ionization detector (FID). The hydrogen production was continued until the biogas volume could not be measured.

3.5. Analytical Methods

The biogas compositions were measured by GC (GC-2014, Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector (TCD) and a 2 m stainless column packed with Unibeads C (60/80 mesh). The operational temperatures of the injection port, column oven and detector were set according to Saraphirom and Reungsang [26]. For the VFAs and alcohol analysis, the effluents were first centrifuged at 6000 rpm for 10 min, then acidified by 0.2 mol/L oxalic acid, and finally filtered through a 0.45 m nylon membrane. The same GC model with a FID and a 30 m × 0.25 mm × 0.25 m capillary column (Stabilwax) was used to analyze the VFAs and alcohol concentrations. Operation conditions were set according to Saraphirom and Reungsang [26]. Total sugar was measured by phenol sulfuric method [35] using glucose as a standard. Total concentrations of nitrogen, phosphorus and VSS were measured according to the standard methods [36]. The hydrogen volume in the biogas was calculated by the mass balance equation [37]. Cumulative hydrogen production was calculated by the modified Gompertz equation [38].

4. Conclusions

The substrate concentration, initial pH and $FeSO_4$ concentration were screened by a Plackett-Burman design as the key factors affecting the production of hydrogen from pineapple waste extract by anaerobic mixed cultures. These variables were optimized by the CCD in order to maximize the P_s and SHPR. The optimum conditions attained were 25.76 g-total sugar/L, initial pH of 5.56 and $FeSO_4$

concentration of 0.81 g/L. Under the optimum conditions, the estimated P_s and SHPR were 5489 mL H_2/L and 77.31 mL H_2/g-VSS h, respectively, which were close to the actual value of 5427 mL H_2/L and 76.43 mL H_2/g-VSS h obtained from the confirmation experiment. The maximum P_s, HY, and SHPR obtained under the optimum conditions were 2.93, 1.75, and 2.93 times higher, respectively, than control. This suggests a significant enhancement of P_s, HY, and SHPR by adding ferrous iron. However, it is important to note that the predicted data are suitable for batch fermentation of hydrogen only and does not necessarily mean that it can always apply to the continuous mode. The addition of Endo–nutrient does not enhance biohydrogen production from pineapple waste extract. The end products were predominantly butyrate and acetate, which generated a butyrate-acetate-type fermentation.

Acknowledgments

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