Acid Pretreatment of Sago Wastewater for Biohydrogen Production

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Abstract. Biohydrogen has been recognized to be one of the future renewable energy sources and has the potential in solving the greenhouse effects. In this study, *Enterobacter aerogenes* (*E. aerogenes*) was used as the biohydrogen producer via dark fermentation process using sago wastewater as the substrate. However, pretreatment of sago wastewater is required since it consists of complex sugars that cannot be utilized directly by the bacteria. This study aimed to use acid pretreatment method to produce high amount of glucose from sago wastewater. Three different types of acid: sulfuric acid (H_2SO_4); hydrochloric acid (HCl) and nitric acid (HNO_3) were screened for the best acid in producing a maximum amount of glucose. H_2SO_4 gave the highest amount of glucose which was 9.406 g/L. Design of experiment was done using Face-centred Central Composite Design (FCCCD) tool under Response Surface Methodology (RSM) in Design Expert 9 software. The maximum glucose (9.138 g/L) was recorded using 1 M H_2SO_4 at 100 °C for 60 min. A batch dark fermentation using *E. aerogenes* was carried out and it was found that pretreated sago wastewater gave a higher hydrogen concentration (1700 ppm) compared to the raw wastewater (410 ppm).

1 Introduction

Hydrogen gas has the potential as a viable alternative fuel and energy carrier of the future since it is a clean fuel with no CO_2 emissions and can be used for generation of electricity. Hydrogen has an enthalpy energy of 122 kJ/g which is 2.75 times greater than hydrocarbon fuels [1]. Hydrogen can be produced chemically and biologically. Indirect biophotolysis, photofermentation, dark fermentation and hybrid system are the examples of biohydrogen process [2]. Dark fermentation is the production of biohydrogen from microorganisms using agricultural waste as the substrate [3]. *E. aerogenes* is a facultative anaerobe hydrogen producing bacteria that can alter the oxygen condition to anoxic condition that recovered the activity of Fe-hydrogenase to produce hydrogen via dark fermentation [4].

In this study, sago wastewater was used instead of sago fibrous pith residues as the alternative substrate for source of glucose production since it contains a high amount of starch [5]. It was reported that about 2.2 mol H_2/mol glucose was produced by *Escherichia*

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coli using sago wastewater as a substrate [6] which showed. Since sago wastewater has complex structure sugars, it needs to be broken down into the simpler fermentable sugar to be utilized by *E. aerogenes*. Two common hydrolysis methods in sago wastewater pretreatment are acid and enzymatic pretreatment. In acid hydrolysis, acid acts as a catalyst that breaks the glycosidic bond into maltose, maltotriose, glucose and dextrin depending on which chain is attacked [7]. In the enzymatic hydrolysis, amylase is used to breakdown the starch into fermentable sugars [8]. However, those reported pretreatments are specifically for sago sago fibrous pith residues and not sago wastewater.

This study was carried out to determine the optimum conditions for the acid pretreatment of sago wastewater in order to obtain maximum fermentable sugar to be used as the substrate by *E. aerogenes* for biohydrogen production.

2 Method

2.1 Collection, preparation and charaterization of sago wastewater

Sago wastewater was collected from a sago mill in Batu Pahat, Johor. The sago wastewater was in brownish color with approximately composed of 60 % water and 40 % sediment. Characterization of sago wastewater was conducted using the standard method for examination of wastewater [9]. Upon use, the wastewater was kept in the cold room to prevent the growth of bacteria while its sediment was sun-dried for 9 hours and then blended into the powder form.

2.2 Screening of acid solution for sago wastewater pretreatment

Three acid solutions that have been chosen for the screening purpose were H_2SO_4, HCl and HNO_3. The aim of the screening was to select the acid that produced the highest amount of glucose from the sago wastewater. Thus, only type of acid solution was varied, while the value of other parameters which were acid concentration (0.5 M), incubation temperature (90 °C) and incubation time (40 min) were kept constant. Sago wastewater samples were used with the composition of 100 mL of sago liquid and 2 g of dried sediment. About 150 mL of 0.5 M of each acid was added into 250 mL conical flask and the solution was stirred well for 1-2 minutes on the hotplate magnetic stirrer at the room temperature. The solution was then placed into the water bath at 90 °C for 40 minutes.

After that, samples were cooled down in the ice water bath and filtered using vacuum filter in order to separate any solid particles from the aqueous solution. NaOH (2 M) solution was added into the filtered solutions for neutralization. The solution was then submerged in the ice water bath to absorb the heat of neutralization and to avoid further decomposition of fermentable sugars [7]. Lastly, glucose concentration was analyzed using dinitrosalicylic acid (DNS) method [10].

2.3 Optimization of pretreatment process parameters

Three parameters of acid pretreatment were chosen to be optimized which are acid concentration (M), incubation temperature (°C) and incubation time (min) with the selected ranges as shown in Table 1. This selection of ranges was varied respectively based on the previous reports [11-13]. Then, the experiment was designed using FCCCD of RSM in the Design Expert 9 software for the analysis of optimum condition for acid pretreatment of sago wastewater. The response was the concentration of glucose (g/L) and all experiments were conducted in triplicates.

Table 1. Experimental range and level of three parameters used in the study.

Factor / Level	Incubation temperature (˚C)	Incubation time (min)	Acid concentration (M)
1	100	90	1.5
0	85	60	1.0
-1	70	30	0.5

2.4 Fermentation of *E. aerogenes* for biohydrogen production

2.4.1 Preparation of E. aerogenes inoculum

The inoculum was prepared aerobically by adding 10 mL of *E. aerogenes* broth in a 250 mL conical flask with 90 mL LB media. The flask was placed in an incubator shaker at 180 rpm and 37°C for 18 hours. The bacteria were actively grown at the exponential phase after 4 hours of inoculation and it was then used as the inoculums or stock culture [14]. Then, the OD of the inoculum was measured at the wavelength of 600 nm using a spectrophotometer.

2.4.2 Media preparation

Two different media that contains the pretreated and raw (non-treated) sago wastewater, respectively were prepared. The raw sago wastewater was prepared by mixing 2 g of sediment with 100 mL of sago liquid. The pretreated sago wastewater was prepared by mixing 2 g sediment with 100 mL sago liquid and the pretreatment process was conducted according to the suggested parameters from the RSM model in the Design Expert 9.0 which were for 88.01 min at 99.9 °C using 1.31 M H_2SO_4 concentration and it was then followed by filtration and pH neutralization as mentioned in Section 2.2. The initial glucose concentration for both media were then measured using DNS method.

2.4.3 Experimental set-up and batch fermentation process

Serum bottles with a capacity of 125 mL were used for the dark fermentation of *E. aerogenes* which was air-sealed and covered with a silicon stopper and aluminium seal cap [15]. An amount of 4 mL inoculum was added to each 76 mL raw and pretreated sago wastewater to make a final working volume of 80 mL. The fermentation was conducted in duplicates at 37 °C with 180 rpm for 48 hours [14]. After 48 hours of incubation, the glass syringe was used to collect the hydrogen gas accumulated in the serum bottle and then the hydrogen analysis was performed. The final glucose concentration in each serum bottle was measured using DNS analysis to observe the glucose conversion after acid pretreatment.

2.4.4 Hydrogen gas analysis

Hydrogen gas was accumulated in the serum bottle after 48 hours. Hydrogen gas concentration for each sample was analyzed using a portable hydrogen gas analyzer and expressed in the unit of part per millions (ppm). If the concentration was more than 1125 ppm, gas dilution was done by taking 10 mL of biogas and mixing it in the 125 mL of empty serum bottle. After that, the concentration of hydrogen was measured again. During gas collection using the glass syringe, the gas pushed up the glass syringe. Thus, the total

volume of biogas equals to the total volume of gas in the serum bottle (45 mL) and gas in the glass syringe. The biogas in the serum bottles consist of carbon dioxide, hydrogen, methane and other gases.

3 Results and Discussions

3.1 Screening of acid solution

It was observed that pretreatment using H_2SO_4 produced the highest glucose concentration which was 9.406 g/L compared to HCl and HNO_3 (Table 2). This result is in agreement with [16] which reported that H_2SO_4 was the best solution for lignocellulosic biomass pretreatment in producing simple sugars. This is due to the ability of diluted H_2SO_4 to effectively remove and recover most of the hemicellulose as dissolved sugars. It was also concluded that H_2SO_4 reached about 72 % dissolution of polysaccharides compared to HCl which was only 41 % dissolution [17]. HNO_3 has also recorded a good conversion of starch to glucose, but its higher price would result in less cost effective in acid recovery for hydrolysis [11]. Based on these reasons, H_2SO_4 was used as the acid solution for optimizing sago wastewater pretreatment parameters.

Table 2. Comparison of glucose concentration after sago wastewater pretreatment using different types of acid at 0.5 M.

Type of acid (0.5 M)	Glucose concentration (g/L)
HCl	6.902
H_2SO_4	9.406
HNO_3	7.615

3.2 Optimization of process parameters for sago wastewater acid pretreatment

Based on the experimental design using FCCCD in Design Expert 9.0, a total of 20 runs were carried out for different ranges of incubation temperature (70, 80 and 100 °C), incubation time (30, 60 and 90 min) and acid concentration (0.5, 1 and 1.5 M). The initial glucose concentration in the raw sago wastewater was measured and its concentration was less than 0.1 g/L. Hence, it can be considered that before pretreatment, sago wastewater contained insignificant amount of glucose. Table 3 lists out glucose concentration (g/L) for all 20 runs that haven conducted. Acid pretreatment at 100 °C for 60 minutes with 1 M H_2SO_4 gave the highest amount of glucose which was 9.138 g/L (Run 13).

Further analysis on the glucose concentration using ANOVA was carried out and the result is summarized in Table 4. It shows that the model given was significant since (Prob>F-value<0.05) and the lack of fit was not significant (Prob>F value>0.1). In addition, the most significant factor affecting the maximum glucose concentration is the incubation temperature where its value of "Probability>F" was 0.0001 which was less than 0.05. It was then followed by acid concentration and incubation time which were less significant. This result can be supported by the previous findings which concluded that at the mild temperature (~120 °C), there was a significant recovery of sugar produced while a higher temperature of pretreatment would cause in the sugar degradation and thus lead to the formation of inhibitors [18].

Table 3. Glucose concentration in sago wastewater after acid pretreatment as a response during optimization using FCCCD.

Run	Incubation temperature (°C)	Incubation time (min)	Acid concentration (M)	Glucose concentration (g/L)
1	85	30	1	2.54
2	85	60	0.5	3.47
3	85	60	1	3.41
4	85	60	1	4.47
5	85	60	1	4.02
6	85	60	1	5.14
7	85	60	1	8.93
8	85	60	1.5	4.68
9	85	90	1	5.84
10	70	90	0.5	0.69
11	100	90	1.5	9.08
12	100	90	0.5	7.00
13	100	60	1	9.13
14	100	30	0.5	4.77
15	100	30	1.5	8.67
16	70	30	0.5	0.48
17	70	60	1	0
18	70	30	1.5	0.33
19	70	90	1.5	1.45
20	85	60	1	6.39

Since incubation temperature is a crucial factor that affects directly to the production of glucose compared to other factors, choosing the appropriate temperature is essential depending on the types of agricultural wastes used in the pretreatment. The "Lack of Fit F-value" of 0.37 implies that the lack of fit condition is not significant and it is a 92.17% chance of the value could occur due to noise. This non-significance lack of fit is good since the model is required to be fitted to the linear regression. The final equation in terms of actual factors is shown in Equation 1.

$$\textit{Glucose concentration} = -18.71970 + 0.23806 \times \textit{Incubation temperature} + 0.02424 \times \textit{Incubation time} + 1.5592 \times \textit{Acid concentration} \quad (1)$$

Although the highest glucose amount has been achieved from acid pretreatment of sago wastewater in Run 13 (Table 3), its process parameters have yet to be optimized. This is based on the 3D response surface model shown in Fig. 1. It depicts that a higher temperature (>100°C) should be used in order to obtain an optimized pretreatment temperature. Monavari *et al*. (2009) [19] proposed that acid pretreatment should be carried out using H_2SO_4 with the concentration range of 0.5 to 1.5 % (w/w) at the temperature of 121-160 °C. From the pretreatment conditions, the yield of sugar from hemicelluloes achieved was 70 % - 95 %.

Table 4. ANOVA Analysis Regression coefficient of predicted models for the response of glucose concentration.

Source	Sum of square	DF	Mean square	F-value	Probability> F	
Model	138.88	3	46.29	20.20	<0.0001	**Significant**
Incubation temperature (°C)	127.51	1	127.51	55.64	<0.0001	
Incubation time (min)	5.29	1	5.29	2.31	0.1483	
Acid concentration (M)	6.08	1	6.08	2.65	0.1230	
Residual	36.67	16	2.29			
Lack of fit	16.44	11	1.49	0.37	0.9217	**Not significant**
Pure error	20.23	5	4.05			
Cor total	175.55	19				

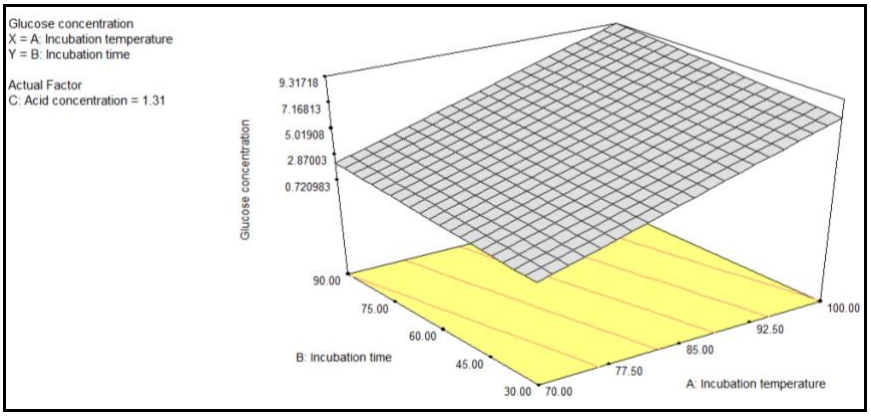

Fig. 1. 3D response surface for model graph.

3.3 Fermentation of *E. aerogenes* for biohydrogen production using pretreated sago wastewater

A batch dark fermentation experiment was carried out to examine the feasibility of pretreated sago wastewater as the substrate for biohydrogen production using *E. aerogenes*. Raw sago wastewater was used as the control. The results shown in Table 5 demonstrates that the pretreated sago wastewater is a potential substrate for *E.aerogenes* to produce biohydrogen. The final concentration of biohydrogen achieved in the media supplemented with pretreated sago wastewater was 1700 ppm (3.59 µmol) which is about 4 times higher compared to the raw sago wastewater. It shows that pretreatment is essential in order to break down the complex sugar into the simplest one so that *E. aerogenes* can easily utilize the sugar to carry out their metabolic activities for biohydrogen production. Previously, it was reported that *E. aerogenes* produced 1.0 mol of hydrogen from 1.0 mol of glucose [20].

Table 5. The final concentration of biohydrogen produced by *E. aerogenes* after 48 hours of incubation.

Substrate	Final concentration of hydrogen (ppm)	Concentration of hydrogen (µmol)
Raw sago wastewater	410	0.81
Pretreated sago wastewater	1700	3.59

4 Conclusions

H_2SO_4 was used in the sago wastewater acid pretreatment since the glucose concentration based on its absorbance showed the highest value compared to HCl and HNO_3. The optimization study of pretreatment process parameters for maximum glucose production using FCCCD was yet to achieve due to the range of temperature used but the highest glucose produced (9.138 g/L) was achieved from run 13 (1 M H_2SO_4 at 100 °C for 60 minutes) with a significant model based the ANOVA analysis. For hydrogen analysis after fermentation, the pretreated sago wastewater achieved was about 4 times higher of hydrogen concentration compared to the raw sago wastewater.

Overall, acid pretreatment sago wastewater is an important step before any agricultural waste can be used as the substrate for fermentation in producing biofuel and biohydrogen. Optimization of important pretreatment parameters such as incubation temperature and concentration of acid is important to improve the yield of glucose from the complex sugars.

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