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PRELIMINARY STUDY ON BIOHYDROGEN PRODUCTION BY *E. coli* FROM SAGO WASTE

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Abstract

Sago waste which has not been fully utilised is a potential substrate for biotechnological applications. This study is concerned with hydrogen gas production by *Escherichia coli (E. coli)* using dark fermentation and sago waste as the substrate. Glucose content after acid pre-treatment process for sago waste at 90°C for 40 minutes was 2.150 g/L. Inoculum size, pH and temperature were the 3 factors investigated during fermentation process. Design of experiment was performed using Central Composite Design (Design Expert v6.0.8). A maximum yield of 2.22 mol H_2/ mol glucose was achieved from Run 8 at the pH of 6, temperature of 40°C and 30% inoculum size.

Keywords: Biohydrogen, *Escherichia coli (E. coli)*, Sago waste, Fermentation.

1. Introduction

Today fossil fuels supply almost 80% of the present world energy demand. Fossil fuels are non-renewable resources and the exhaustion of this resource of energy has brought concerns to many people. Presently, the utilisation of fossil fuels are causing global climate change mainly due to the emission of pollutants like CO_x, NO_x, SO_x, C_xH_x, soot, ash, droplets of tars and other organic compounds, which are released into the atmosphere as a result of their combustion [1]. There have been grown in fears of greenhouse gas emissions and other critical environmental issues over the usage of fossil fuels. As the cure of the depletion of fossil fuels and their environmental wrongs, renewable, sustainable and carbon-neutral energy production is needed.

Studies found that one of the attractive ways is to produce biofuel by using

Nomenclatures

Greek Symbols

μ	Specific growth rate, h^{-1}

Abbreviations

DNS	3, 5-dinitrosalicylic acid
HHV	Higher heating value, MJ kg^{-1}
LB	Luria-Bertani medium

biological process. Among the various biofuel options, biohydrogen gas is an attractive future energy carrier since it has higher efficiency of conversion to usable power, low to non-existent generation of pollutants and high energy density [2]. Hydrogen is also being claimed as an excellent energy carrier as it has a high calorific value (HHV of 142 MJ kg^{-1}) and has low emissions at the point of use [3]. The current hydrogen gas production is unfavourable as it is being generated from fossil fuels through thermo-chemical processes, such as hydrocarbon reforming, coal gasification and partial oxidation of heavier hydrocarbons [4]. Therefore production of hydrogen gas by using biological means has gaining attentions of many researchers due to its potential for inexhaustible, low-cost and renewable source of clean energy.

Biohydrogen production is an entropy reducing process, which could not be done by mechanical or chemical systems. Moreover, biological methods are well suited for decentralised energy production in small-scale installations in locations where biomass or wastes are available, thus avoiding energy expenditure and costs for transport [4]. Recently, studies have focused majorly in fermentative hydrogen production by using waste materials. Hydrogen fermentation is favorable since hydrogen could be produced at higher rate and the process can be carried out on numerous organic wastes and wastewaters enriched with carbohydrates, thus achieving sustainable low cost biohydrogen production with concomitant waste purification [4].

Different microorganisms were reported to participate in the biological hydrogen generation system such as green algae, cyanobacteria (or blue-green algae), photosynthetic bacteria and fermentative bacteria such as *Enterobacter aerogenes, Escherichia coli (E. coli)* and *Clostridium butyricum.* These microorganisms are able to evolve molecular hydrogen by means of a hydrogenase [1].

The existing energy supply is heavily dependent on fossil fuels, which are geographically unevenly concentrated in the world and may soon deplete. Moreover, the production and consumption of fossil fuels are closely linked to the deterioration of environment and severely threaten human health and affect the climate and ecological balances [5]. Therefore a new source of energy comes from renewable resources with minimal impact on environment as well as human health is needed. As a clean energy, producing only water after combustion, hydrogen is alternative fossil fuels in the future.

Producing hydrogen biologically by means of dark fermentation is indeed an impressive solution to overcome the matter regarding diminution of fossil fuel. It may be harvested during biological treatment of waste, the organic in which serve as carbon and energy sources for the microbes. Sago waste is one of the remarkable wastes that is worth a try in producing biohydrogen, where

it is able to give high starch yields (15-25 ton/ha of dry starch) [6]. Moreover sago starch costs 15-30% less to produce per acre than other starch such as corn starch making sago an alternate and strategic source of renewable energy.

This study is aimed to determine the suitable fermentation conditions of biohydrogen producing microbes which was *E. coli* using pre-treated sago waste as the substrate. The sago wastewater with pH of 4 underwent pre-treatment process for glucose conversion to be readily used by the bacteria. Furthermore, the correlation between cell growth and biohydrogen production using *E. coli* was also investigated.

2. Experimental Method

2.1. Sample collection

Sago wastewater contains a large amount of starch at pH of 4 was retrieved from a sago process plant in Riau, Indonesia (PT National Sago Prima). The sago wastewater was filtered to remove any large solid particles before use.

2.2. Pre-treatment process

Pre-treatment was done by using 0.5 M sulphuric acid (H_2SO_4). An amount of 60% (v/v) of sago wastewater was mixed with 150ml of 0.5M concentrated sulphuric acid [7]. The solution was stirred in 1-2 minutes and was then placed into a water bath with the temperature set to 90°C for 40 minutes. The solution was let cooled in ice water to allow settling. Then, filtration was done to remove any solid particles. The hydrolysate was neutralised with 0.5M sodium hydroxide (NaOH). The solution was then submerged in ice water bath to absorb the heat of neutralisation to avoid further decomposition of reducing sugar. The total reducing sugar in hydrolysate was analysed by using spectrophotometer (540 nm) using a reducing chemical reagent, 3,5-dinitrosalicylic acid 98 % solution (DNS reagent).

2.3. Bacterial culture

E. coli were cultured on Petri plates using agar supported Luria-Bertani (LB) medium. The plates were incubated at 37°C, and after 24 hours of growth, the fresh colonies were used to prepare the inoculum. For inoculum preparation, the seed cultures were prepared aerobically in a 50ml flask (working volume was 30 ml) containing LB medium and then the flasks were placed in a shaking incubator at 250 rpm agitation rate (37°C, 12 h) [8].

2.4. Fermentation

Fermentation process was done in 500 ml shake flask with the working volume of 300 ml. The culture temperature was controlled at 28 to 40 °C and pH of 6.0- 8.0 was maintained throughout the fermentation process. The fermentation was run for 14 hours and sampling (in triplicate) was taken every 2 hours for parameter analysis.

2.5. Analysis

2.5.1. Glucose analysis using DNS method

Sample with a volume of 0.5 ml was pipetted into the test tube and the volume was equalised to 3 ml with water. Then 3 ml of DNS reagent was added and the content was heated in a boiling water bath for 5 minutes. While the content of the tube was still warm, 1 ml of 40% Rochelle salt solution was added. The sample was then cooled and absorbance was measured at 540 nm using spectrophotometer. The concentration of the glucose was calculated from glucose standard curve which was already generated prior to the analysis.

2.5.2. Hydrogen gas analysis using hydrogen gas analyser

Hydrogen is a gas with no color or smell. It has a neutral pH that does not show on litmus paper. Therefore for hydrogen gas detection in this experiment, hydrogen gas analyser was used. The concentration of the hydrogen gas detected was shown in the unit of part per millions (ppm).

2.6. Experimental set-up

The experimental set-up of fermentation process of for biohydrogen production is represented in Fig. 1.

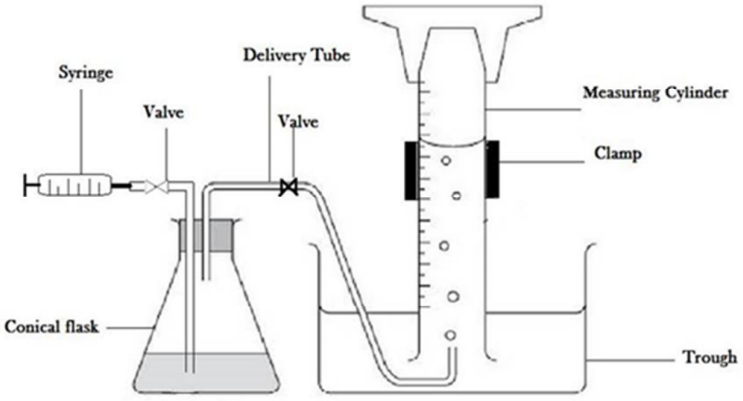


Fig. 1. Experimental set-up of fermentation process for biohydrogen production.

2.7. Design of experiment

Table 1 shows the design of experiment for analysis of optimum fermentation condition for biohydrogen production by *E. coli*. The design of experiment was done using Central Composite Design in the Design Expert software (v6.0.8). For analysis of experiment, three factors have been chosen which were temperature (28 °C- 40 °C), pH (6-8) and inoculum size (10% - 30% v/v).

Table 1. Design of experiment of optimisation of fermentation conditions.

Run	Factor 1 A: pH	Factor 2 B:Temperature (°C)	Factor 3 C: Inoculum size (% v/v)
1	6.00	28	10
2	6.00	28	30
3	8.00	28	10
4	7.00	34	30
5	7.00	34	20
6	8.00	40	10
7	7.00	40	20
8	6.00	40	30
9	8.00	28	30
10	7.00	34	20
11	7.00	34	20
12	8.00	40	30
13	7.00	34	10
14	6.00	40	10
15	7.00	34	20
16	7.00	28	20
17	8.00	34	20
18	6.00	34	20
19	7.00	34	20
20	7.00	34	20

3. Results and Discussion

3.1. Pre-treatment of sago waste

Pre-treatment is an important procedure in converting complex sugar into simple sugar to enhance fermentation process. *E. coli* is a bacterium that most likely will consume glucose before any other sugar.

Glucose level of sago starch was measured before performing pre-treatment to compare the glucose content before and after the process. DNS method was used to analyse the glucose content. The glucose content of raw sago wastewater measured was found to be lower compared to sago wastewater after pre-treatment process as shown in Table 2.

Table 2. Glucose content in raw and pre-treated sago wastewater.

	Glucose content (g/L)
Raw Sago Wastewater	1.153
Pretreated Sago Wastewater	2.150

The results showed that glucose content of pre-treated sago wastewater was higher than raw sago wastewater by approximately 86%. In acid hydrolysis, the acid acts as the catalyst to break the starch's glycosidic bonds to produce dextrin, maltotriose, maltose and glucose depending on the relative location of the bond under attack as counted from the end of the chain [9]. Therefore after the pre-treatment process, glucose content in the sago wastewater had increased and this will enhance fermentation process for biohydrogen production.

3.2. Fermentation process

3.2.1. Optimisation of the production of biohydrogen gas

Optimisation study was carried out using pre-treated sago wastewater as the raw material and *E. coli* as the hydrogen producing bacteria. Three factors have been chosen for the optimisation studies which were pH, temperature and inoculum size of the fermentation in order to generate maximum concentration of hydrogen gas.

A total of 20 runs (Table 1) were conducted with parameters suggested by Design Expert software. As illustrated in Fig. 2, after 14 hours of fermentation process, run 8 with fermentation conditions of pH 6, temperature of 40°C and 30% inoculum size achieved the highest hydrogen yield which was 53 ppm or 2.22 mol H_2/mol glucose. The yield obtained was comparable with hydrogen production by other substrate. According to Argun & Kargi [10], their study obtained the highest hydrogen yield, 2.7 mol H_2/mol glucose by using sweet potato starch with co-culture of *Clostridium butyricum* and *Enterobacter aerogenes*. Another finding by Bakonyi et al. [11] reported that an amount of 3.37 mol H_2/mol glucose was achieved by using genetically modified *E. coli* strains.

The fermentation medium with pH of 6 was one of the conditions testified to contribute to a high yield of hydrogen gas. As reported by Fang & Liu [12], the highest hydrogen gas production at pH 5.5 to 6 was found whereas further increase of the pH would decrease the production of hydrogen. Moreover the optimum pH range to support the growth of *E.coli* is between 6 to 7 since at this pH range, the bacteria's growth is maximum and so the hydrogen production. However, low pH can be used to inhibit methanogens and thus improve hydrogen gas production [12]. This is because the presence of methane would decrease hydrogen content.

In this study, 40°C is the temperature that gave the highest yield of hydrogen gas. As has been claimed by Pakarinen [13], a higher hydrogen yield at higher temperature could be expected given the fact that higher temperatures favour hydrogen formation but not hydrogen-consuming reactions such as methane. Moreover, Luo et al. [14] stated that hydrogen yield from cassava stillage was enhanced from 14 to 70 ml gVS^{-1} when the temperature was increased from 37 to 60°C. *E.coli* can grow with a maximum of 49°C but the optimal growth is at 37°C to 40°C [15]. With optimal growth of *E.coli* at this temperature, the production of hydrogen will also be at a maximum rate. Above the temperature of 42°C, the growth of *E.coli* will become slower as the activity of the enzyme in the cell is abruptly lowered to a characteristic new level [11].

High inoculum size which was 30% (v/v) in this study generated the highest yield of hydrogen gas. Large inoculum size can be used to inhibit methanogens

that might lower the production of hydrogen gas [13]. Furthermore, as reported by Seengenyoung et al. [16], inoculum concentration of 10, 20 and 30% gave 78.5, 82.4 and 82.6% total carbohydrate removal for hydrogen production, respectively. It showed that with high total carbohydrates removal, hydrogen yield would also be higher.

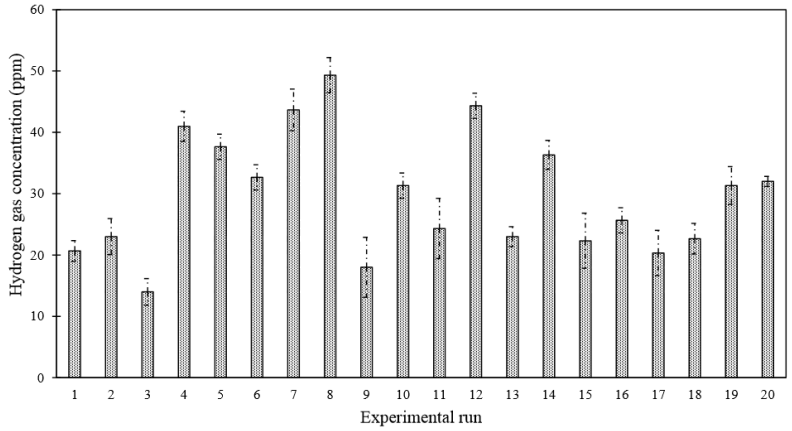


Fig. 2. Hydrogen gas production by *E. coli* with respect to 20 runs (error bars indicate standard deviation between triplicates).

As mentioned before, pH, temperature and inoculum size of fermentation were chosen for the optimisation studies in order to produce maximum hydrogen gas from model elaborated response. However, the optimisation of process conditions from this study was not achieved. Consequently, the result only showed the maximum hydrogen production but not the optimised one. The quadratic relating to the glucose concentration with independent variables A, B and C are as follows Eq. (1):

Hydrogen gas production

$$=32.39-2.90A+11B+5.10C-6.73A^2+2.77B^2+2.27C^2-0.50AB-1.25AC+2 \quad (1)$$

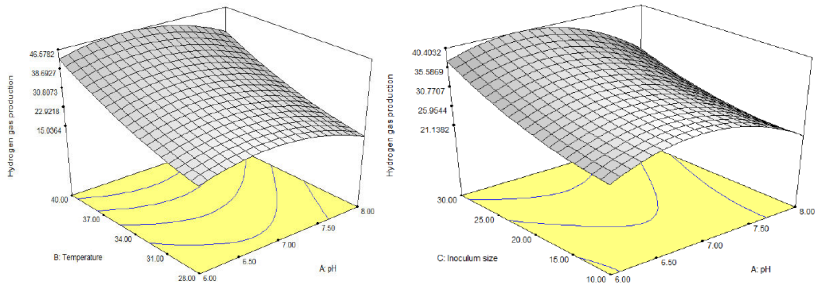
From the statistical analysis in Table 3, the overall ANOVA for response surface for quadratic model demonstrated a significant model with *p*-value equals to 0.0001. Variables A, B and C are significant model terms. The variables that are significant to each other are pH, temperature and inoculum size.

To investigate the effects of each independent variable on the hydrogen production, the 3D response surface and contour plots response surface were implemented. The maximum predicted value can be referred by the surface defined in the smallest ellipse in the contour diagram. Fig. 3(a) and (b) illustrate the response surface described by the model equation to estimate maximum hydrogen production over independent variables. Both contour plots suggested that high hydrogen yield can be obtained at high temperature and inoculum size at any pH. Figure 3(c) shows that factor interactions between inoculum size and temperature gave higher hydrogen yield and further study correlated to these two factors is necessary to obtain optimum parameter for maximum hydrogen production.

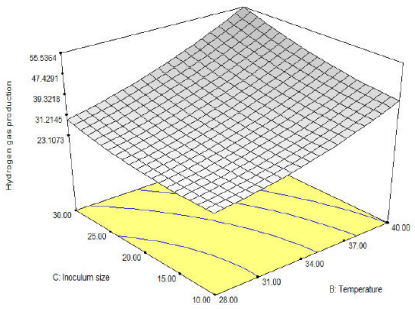
Table 3. ANOVA for response surface quadratic model.

Source	Sum of Square	Degree of Freedom	Mean Square	F-Value	*p*-value Prob > F	
Model	1725.40	9	191.71	14.80	0.0001	significant
A	84.10	1	84.10	6.49	0.0290	N/A
B	1210.00	1	1210.00	93.40	< 0.0001	N/A
C	260.10	1	260.10	20.08	0.0012	N/A
A^2	124.45	1	124.45	9.61	0.0113	N/A
B^2	21.14	1	21.14	1.63	0.2303	N/A
C^2	14.20	1	14.20	1.10	0.3197	N/A
AB	2.00	1	2.00	0.15	0.7026	N/A
AC	12.50	1	12.50	0.96	0.3491	N/A
BC	32.00	1	32.00	2.47	0.1471	N/A
Residual	129.55	10	12.95	N/A	N/A	N/A
Lack of Fit	66.05	5	13.21	1.04	0.4833	not significant
Pure Error	63.50	5	12.70	N/A	N/A	N/A
Core Total	1854.95	19	N/A	N/A	N/A	N/A

R-Sq= 93.02% R-Sq (adj)= 86.73%



(a) (b)



(c)

Fig. 3. Response surfaced described by the model equation to estimate maximum hydrogen production over independent variables; (a) fermentation temperature and pH, (b) pH and inoculum size, (c) temperature and inoculum size.

3.2.2. Growth culture of *E. coli* correlated with hydrogen gas production

To determine the correlation of cell growth with hydrogen production, the data used for this study was taken from run 8 (pH 6, 40°C and 30% inoculum size) as it achieved the highest hydrogen gas yield. Cell growth and hydrogen production was monitored hourly. The sampling was done in triplicate. Cell concentration and hydrogen gas production with respect to time were plotted and shown in Fig. 4.

The results showed that bacterial growth followed a similar pattern with hydrogen production, as illustrated in Fig. 4. Cells grew exponentially during the first hour until the twelfth hours. The specific growth rate, μ calculated from the slope during exponential phase was 0.0212 hr^{-1}.

It can be also be deduced from Fig. 4 that during exponential phase, hydrogen gas began to produce and kept increasing until the fourteenth hour. Therefore, it can be said that during exponential phase, where the cells divided rapidly, they were also emitted hydrogen gas at the same time or in simpler words, cell growth and hydrogen production occurred simultaneously.

The hydrogen gas produced was measured through the water displacement in a measuring cylinder. The growth of cells as well as hydrogen gas production began to decline when approaching 14th hours of the fermentation time. Hydrogen is a primary metabolite which is associated with cell growth [17]. Gas production began within the first 12 hours of fermentation during the exponential phase in which pH became lowered during this phase by the formation of organic acids affecting microorganism membrane load and hydrogenase activity. This inhibited hydrogen production during the stationary phase [17].

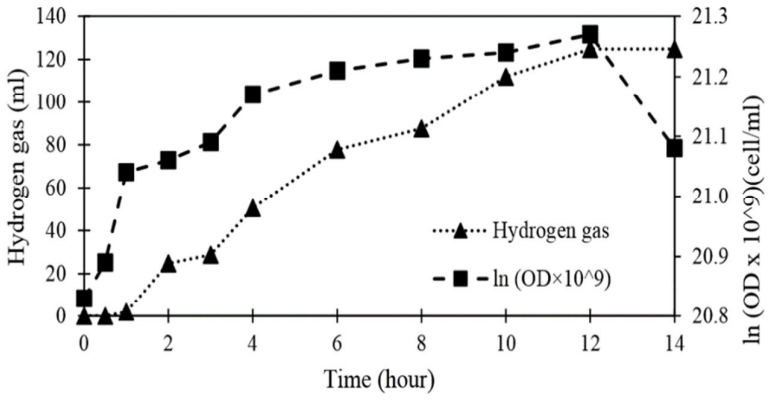


Fig. 4. *E. coli* cell growth curve and hydrogen gas production with respect to time for run 8 (pH 6, 40°C and 30% inoculum size).

4. Conclusions

Studies on pre-treatment process of sago waste and fermentation process for hydrogen production by using *E.coli* were conducted. Pre-treatment of sago waste was performed at the temperature of 90°C for 40 minutes using 0.5M acid concentration. The concentration of reducing sugar obtained was 2.150 g/L with approximately 86% conversion of reducing sugar. Pre-treatment is important to

make glucose readily available for *E. coli* consumption since they prefer glucose than any other carbon sources. The highest yield of hydrogen obtained was 2.22 mol H_2/ mol glucose at pH 6 and 40°C with 30% (v/v) of inoculum size. *E. coli* cell growth and hydrogen production occurred concurrently. The findings of this study can serve as a starting point for using sago waste as the substrate for biohydrogen production by bacterial cultures.

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