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STATISTICAL SCREENING OF PHYSICO-CHEMICAL FACTORS FOR BIOHYDROGEN PRODUCTION BY *ENTEROBACTER AEROGENES* FROM SAGO WASTEWATER

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Abstract

High organic content of sago wastewater (SWW) can be utilized as a substrate for biohydrogen production. After acid pre-treatment of SWW using 1.5% sulfuric acid at 121ºC for 60 minutes, its glucose content increased from 0.146 g/L to 9.36 g/L. In this study, *Enterobacter aerogenes* (*E. aerogenes*) was used as the biohydrogen producer. Screening of ten physico-chemical factors that influenced biohydrogen production were performed using Plackett-Burman design tool (Design Expert 9.0). Yeast extract concentration, fermentation temperature, and inoculum concentration were ranked as the three most significant factors. The maximum hydrogen concentration, volume production, and hydrogen yield achieved in this study were 454.30 µmol/L, 88 mL, and 2.42 mmol H_2/mol glucose, respectively. The findings from this study can be used for further optimization of process conditions for biohydrogen production by *E. aerogenes* using SWW as the substrate.

Keywords: Biohydrogen, *Enterobacter aerogenes*, Plackett-Burman design, Renewable energy, Sago wastewater.

1. Introduction

As the primary source of energy, the global demand of fossil fuel increased exponentially. Since it is non-renewable, the current oil reservoir will not be sufficient to fulfil the energy demand. Hydrogen energy serves as an alternative to a more sustainable energy. Hydrogen is considered as a clean fuel because it produces only water as a byproduct [1]. According to Kapdan and Kargi [2], hydrogen energy is almost three times greater than hydrocarbon fuels. However, the conventional method to produce hydrogen is still generated from fossil fuels [3]. Therefore, hydrogen production by biological means has attracted significant attention from the researchers due to the unlimited, inexpensive and renewable source of clean energy [4].

Sago plant is amongst the primary commodities in Malaysia [5]. As previously reported, there was about 47000 MT (megatons) of dry starch exported form sago industry in 2015 [6]. Annually, about 2.5 million tonnes of effluent or known as sago wastewater (SWW) are generated from this industry [7]. It is reported that high organic loading (COD and BOD) of SWW had caused severe threat to the water ecosystem [8]. Unfortunately, there is no appropriate treatment of the SWW when it is discharged to the river. Hence, the detrimental effect of SWW is inevitable. Therefore, the major challenge is how to utilize sludge or waste to produce a useful and viable product. Previous investigations have shown that sago waste was used as a substrate to produce *Spirullina* [9], bioethanol [10], and methane [11]. Alternatively, SWW can be used as a raw material for biohydrogen production via dark fermentation [11-13]. Dark fermentation by bacteria is one of the methods that commonly used to produce biohydrogen from waste [12]. In this scenario, the ultimate goal in reducing environmental side effect of SWW can be attained.

It is reported that temperature, pH and inoculum concentration are among the physical factors which affect the yield of biohydrogen [14]. The bacterial growth rate during biohydrogen production was maintained at the optimum pH and temperature. Meanwhile, the size of inoculum determined the biohydrogen production rate [15].

According to previous research, biohydrogen was produced when the bacteria was in the mid-log phase until stationary phase [4]. Other than physical factor, chemical content of fermentation media also contributes in the optimization of biohydrogen production [12, 16]. Metal ion plays significant role as a cofactor for enzyme activity and as a component of metal complexes, such as Fe and Mg [15, 17, 18] which are needed as an intermediate donor [19] and cofactor for major glycolytic enzymes [20] during biohydrogen generation, respectively. It was previously reported that SWW has high starch amount but low nitrogen content and hence it needs supplementation of nitrogen if to be used as the substrate of fermentation [21]. Usually, yeast extract and malt extract are supplemented to the media as the nitrogen and vitamins sources [22]. Yeast extract presence is significant because it serves as the nitrogen source and the growth factors for bacteria [23].

Hence, this paper aimed to screen 10 physico-chemical factors (SWW concentration, temperature, pH, inoculum concentration, malt extract, yeast extract, iron, magnesium, copper, and nitrogen sparging) affecting biohydrogen production using SWW as a sole substrate during the dark fermentation of *E. aerogenes*. Plackett-Burman design tool in Design Expert Version 9.0.6.2 (Stat. Ease. Inc., USA) software was employed for designing the experiment and screening purposes.

2. Methods

2.1. Microorganism and inoculum condition

In this study, *E. aerogenes* from DSMZ, Germany (strain reference: CDC 819-56) was used as biohydrogen producer. The strain was grown in agar media (Luria-Bertani agar) at the temperature of 37°C for 24 hours inside an incubator. Inoculum stock was then prepared by transferring a loopful of fresh colony into 100 mL of LB medium in a flask and placed in an incubator shaker. The inoculum was agitated for 18 hours (rotational speed of 150 rpm, temperature of 35°C), until the optical density (OD600) of bacteria reached 4.5 [24].

2.2. Preparation of the substrate

In this study, the SWW was obtained from Johor, Malaysia. COD (chemical oxygen demand), pH, VSS (volatile suspended solid), TSS (total suspended solid),and TDS (total dissolved solid) were measured to determine the characteristic of the raw material [25]. To sterile the raw material, the SWW was autoclaved for 20 minutes (temperature of 121°C). The SWW was then stored in a cold room (4°C) for future use [24]. The starch of SWW was hydrolysed using acid pre-treatment method. Fresh SWW was mixed with the acid (1.5% (v/v) pure sulfuric acid (98%)) and then autoclaved again at 121°C for 60 min [24]. After the pre-treatment process, hydrolysed SWW was cooled in the ice bath. NaOH pellet was then used to neutralize the acidic pre-treated SWW to the pH according to design of experiment. Lastly, the cloth coffee filter was used to filter the media from any particles and sediments.

2.3. Preparation of fermentation media

In order to support the growth of bacteria, the fermentation media was fortified by some endonutrients; K_2HPO_4 (7.0 g/L), KH_2PO_4 (5.5 g/L), $(NH_4)_2SO_4$ (1.0 g/L), $CaCl_2.2H_2O$ (0.021 g/ L), $Na_2MoO_4.2H_2O$ (0.12 g/L), nicotinic acid (0.02 g/L), Na_2SeO_3 (0.172 mg/L), $NiCl_2$ (0.02 mg/L) [12]. Next, the media was autoclaved at the temperature of 121ºC (duration of 15 min long) for sterilization. Throughout this experiment, the fermentation media was placed in the serum bottle (size 125 mL) with 80 mL of working volume [24].

2.4. Preparation of the substrate

The ten chosen physico-chemical factors include SWW concentration, temperature, pH, inoculum concentration, yeast extract, malt extract, iron, magnesium, copper, and nitrogen sparging (Table 1). Fermentation was performed using the parameter conditions referring to the Plackett-Burman design generated by Design Expert version 9.0.6.2 (Stat. Ease. Inc.) which resulted in 12 runs of experiment (Table 2). Under sterile condition, inoculum concentration, malt extract, yeast extract, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, and $CuCl_2.2H_2O$ were supplemented to the fermentation media. For SWW composition, the concentration was varied to 50% and 100%. In order to make 50% SWW, 50% volume of SWW was mixed with 50% volume of distilled water. The bottle was then sparged by pure nitrogen gas. After it was ready, a silicone stopper and aluminium cap was used to tightly seal the bottle. Experiments were performed to obtain the level of significance of each factor to the hydrogen concentration (μmol/L). All the bottles were then agitated

for 48 hours (rotational speed of 150 rpm) in an incubator shaker (Infors, AG CH-4103 Bottmingen).

Table 1. The codes and levels of variables.

Code	Variable	Low level	High level	Reference
X_1	**SWW concentration (%)**	50	100	[7, 13]
X_2	**pH**	5.5	7.5	[26]
X_3	**Temperature (˚C)**	35	40	[27, 28]
X_4	**Inoculum concentration (%)**	5	15	[28, 29]
X_5	**Malt extract (g/L)**	0	2	[22]
X_6	**Yeast extract (g/L)**	0	2	[24]
X_7	**$FeSO_4.7H_2O$ (mg/L)**	100	300	[22, 30]
X_8	**$MgSO_4.7H_2O$ (mg/L)**	100	300	[27]
X_9	**$CuCl_2.2H_2O$ (mg/L)**	2	10	[7]
X_{10}	**Nitrogen sparge (minute)**	0	3	[31]

Table 2. Plackett Burman experimental design for evaluation of 10 physico-chemical factors for biohydrogen production.

Run	X_1 (%)	X_2	X_3 (ºC)	X_4 (%)	X_5 (g/L)	X_6 (g/L)	X_7 (mg/L)	X_8 (mg/L)	X_9 (mg/L)	X_{10} (min)
1	100(+)	5.5(-)	35(-)	5(-)	0(-)	0(-)	300(+)	300(+)	10(+)	0(-)
2	100(+)	7.5(+)	40(+)	15(+)	0(-)	0(-)	100(-)	300(+)	10(+)	3(+)
3	50(-)	7.5(+)	40(+)	5(-)	2(+)	0(-)	100(-)	100(-)	10(+)	3(+)
4	100(+)	5.5(-)	40(+)	15(+)	0(-)	2(+)	100(-)	100(-)	2(-)	3(+)
5	100(+)	7.5(+)	35(-)	15(+)	2(+)	0(-)	300(+)	100(-)	2(-)	0(-)
6	100(+)	7.5(+)	35(-)	5(-)	2(+)	2(+)	100(-)	300(+)	2(-)	0(-)
7	50(-)	7.5(+)	40(+)	15(+)	0(-)	2(+)	300(+)	100(-)	10(+)	0(-)
8	50(-)	5.5(-)	40(+)	15(+)	2(+)	0(-)	300(+)	300(+)	2(-)	3(+)
9	50(-)	5.5(-)	35(-)	15(+)	2(+)	2(+)	100(-)	300(+)	10(+)	0(-)
10	100(+)	5.5(-)	35(-)	5(-)	2(+)	2(+)	300(+)	100(-)	10(+)	3(+)
11	50(-)	7.5(+)	40(+)	5(-)	0(-)	2(+)	300(+)	300(+)	2(-)	3(+)
12	50(-)	5.5(-)	35(-)	5(-)	0(-)	0(-)	100(-)	100(-)	2(-)	0(-)

2.5. Microorganism and inoculum condition

2.5.1. Hydrogen sampling and concentration measurement

After 48 hours, hydrogen gas was formed, and the volume of gas produced was recorded. A 50 mL volume of gas tight syringe (Agilent 5190-1547) was used to collect the gas accumulated in the headspace (volume of 45 mL) of serum bottle [13]. In order to measure its concentration, 1 mL of hydrogen gas was injected to the hydrogen analyser (Portable Gas Detector GRI-8310) [13]. In the case when too much gas was produced, it resulted in the gas build-up pressure which pushed up the gas tight syringe. Hence, the total volume of gas is the volume of headspace and gas in the syringe due to this pressure.

2.5.2. Determination of glucose concentration

Initially, the fermented media was filtered using nylon filter (Nylon6, 0.45μm) connected to the syringe in a 1 mL vial (Ioflow). The initial and final glucose concentration in the fermented media was determined using High Performance Liquid Chromatography (HPLC) analysis [13]. Glucose concentrations of 0.5 g/L, 1 g/L, 5 g/L, 10 g/L, and 50 g/L were used to plot a standard curve. Both mobile phase and glucose standard solutions were filtered using a vacuum pump (Fisherbrand, FB70155) connected to the filter set with a nylon membrane filter (Ioflow 0.45μm). A linear fit equation was derived from the standard curve, and it was used to calculate the glucose concentration. The reading of the bacterial growth was also quantified using colony cell counting method [32].

3. Results and Discussion

3.1. Characteristic of SWW

The characteristic of SWW from this study has previously been determined as presented in Table 3 [13]. It was found that SWW was acidic in which it has a low pH (3.74). Moreover, TSS, COD, and total carbohydrate from SWW were found to be 14000 mg/L, 23700mg/L, and 2781 mg/L, respectively. These numbers were higher as compared with the same sample from the previous study [7]. It suggested that SWW sediment contains high organic compound. Glucose content before pre-treatment was found to be 0.146 g/L. After sulfuric acid pre-treatment the complex sugar has been successfully broken down which resulted in high glucose content (9.36 g/L). So far, acid pre-treatment is found to be more effective than heat pre-treatment, despite volatile fatty acid (VFA) such as acetate that may also produce as the by-product [33]. Acetate can cause detrimental effects to the metabolism of bacteria if the concentration is within the range of 4-10 g/L [34]. It was found from this study that the acetate concentration after acid pre-treatment was lower than the range (0.1 g/L), indicating that the growth of bacteria will not be inhibited.

Table 3. Comparison of SWW's characteristic in this study and other findings [13].

Parameter	Present study	[7]	[35]
pH	3.74	4.2	5.2
COD [mg/L]	23700	10640	2080
TDS [mg/L]	1017	*NR	2010
TSS [mg/L]	14000	1998	86
VSS [mg/L]	39.5	NR	*NR
Total carbohydrate [mg/L]	2781	557	*NR

*NR: Not reported

3.2. Screening of factors affecting biohydrogen production based on Plackett-Burman design

Plackett-Burman design was used to identify the key factors with respect to the hydrogen production [36]. Plackett-Burman design assumed that no interactions between different factors occur in the range of variables under consideration [37]. The result of factor screening is given in Fig. 1. Out of 12 runs, run 10 achieved the highest hydrogen production compared to others. The hydrogen concentration, gas

volume, hydrogen yield and glucose conversion were 454.30 µmol/L, 87 mL, 2.42 mmol H_2/mol glucose and 4.42 mmol H_2/mol glucose consumed, respectively. While for the control variable where glucose was used as the carbon source, the hydrogen concentration and yield were 23.77 µmol/L and 0.43 mmol H_2/mol glucose, respectively. The yield (2.42 mmol H_2/mol glucose) obtained in this study was slightly lower than the previous finding, where glucose was used as the sole carbon source with the yield of 3.1 mmol H_2/mol glucose [26]. This indicates that SWW has a potency to provide glucose during the fermentation.

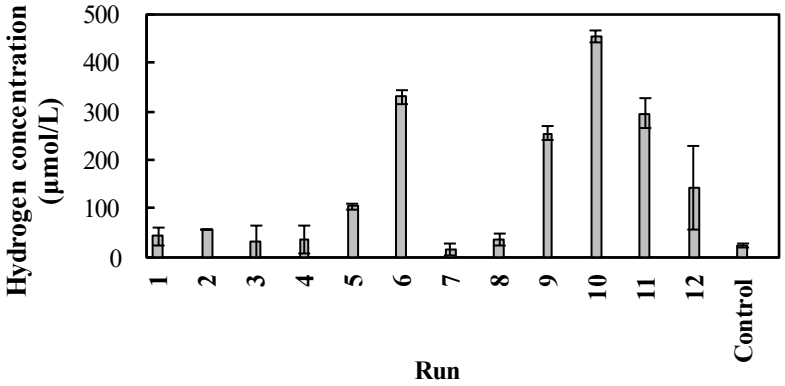

Fig. 1. Different concentrations of hydrogen produced from the total 12 runs of Plackett-Burman Design.

The summary of ANOVA is given in Table 4. ANOVA is an analysis to measure the significance of a model. The application is widely used to test the experimental data for a proposed model [38]. The mean square is obtained by dividing sum square with degree of freedom. The Fisher variation ratio (*F-value*) measures how good the factor describes the data variation of its mean. Meanwhile, the *p-value* determines the significance level of each coefficient. It is considered as significant if *p-value*<0.05 [39]. Therefore, in this study, fermentation temperature (0.0025), inoculum concentration (0.0029), yeast extract (0.0009) and malt extract (0.0098) were the significant factors with a confidence interval above 95%. The R^2 was high (0.922) indicating that the model is suitable to represent the data from this experiment. The model for this experiment is given by Eq. (1).

$$H_2 = 1167.20 - 27.15C - 13.21D + 52.0E + 81.20F \quad (1)$$

where hydrogen concentration (H_2) is the function of temperature (C), inoculum concentration (D), malt extract (E) and yeast extract (F).

The comparison of relative strength effect of selected factors on hydrogen production is presented in Fig. 2. Main effect value indicates the level of significance of the factor. From the 10 selected factors, the level of significance in the decreasing rank were yeast extract (+12.99), temperature (-10.86), inoculum (-10.5), malt extract (+8.33), sago waste (+3.25), Mg (+3.09), pH (-1.77), Fe (+1.32), Cu (+1.21), and nitrogen sparge (+0.27). Positive value of the main effect indicates that a higher value is more preferred for the respective factor. In contrary, negative main effect suggested that low value of the respective factor is the most preferable. For this study, yeast extract, temperature, and inoculum concentration

are the three most influential factors. The model suggested the optimized solution for hydrogen concentration under physical condition for temperature, inoculum concentration, and pH of 35.003°C, 5%, and 6.5, respectively, with concentration of malt extract, yeast extract iron, copper, and SWW of 2 g/L, of 2 g/L, 200 mg/L, 6 mg/L, and 50%, respectively. This resulted in the hydrogen concentration of 416.25 µmol/L.

Table 4. ANOVA for selected factorial model.

Analysis of variance table [Partial sum of squares - Type III].

Source	Sum of Squares	df	Mean Square	*F Value*	*p-value* Prob > F
Model-significant	2.19×10^5	4	54804.83	20.87	0.0005
C-Temperature	55263.96	1	55263.96	21.04	0.0025
D-Inoculum concentration	52328.52	1	52328.52	19.92	0.0029
E-Malt extract	32511.41	1	32511.41	12.38	0.0098
F-Yeast extract	79115.45	1	79115.45	30.12	0.0009
Residual	18386.42	7	2626.63		
Cor Total	2.376×10^5	11			

R^2=0.9226, Adj R^2=0.8784, Pred R^2=0.7726, Adeq Precision=13.004

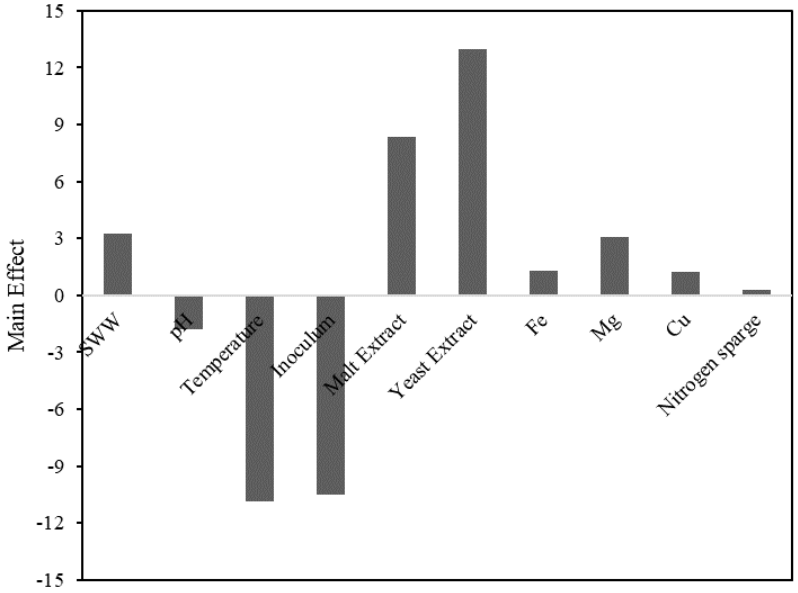

Fig. 2. Different concentrations of hydrogen produced from the total 12 runs of Plackett-Burman Design.

Even though the value of the true yield of hydrogen from SWW is comparable with the prior study [26], it was still too low relative to the maximum theoretical yield. Theoretically, *E. aerogenes* is capable to produce maximum 2 mol H /mol glucose [4]. As comparison, 2.2 mol H_2/mol glucose was successfully obtained from SWW using *E. coli* as a seed [13]. Another finding which investigated the effect of supplementation of media to distillery effluent as substrate for biohydrogen production using distillery effluent as a substrate, found that under the optimum condition (yeast extract 2.46 g/L, malt extract 1.28 g/L, $FeSO_4$ 260 mg/L, $MgSO_4$ 2.35 g/L and $CuCl_2$ 3.94 mg/L) there was about 1.42 mol H_2/mol glucose of hydrogen produced [22].

3.3. Effect of yeast extract on the concentration of hydrogen

Bacteria need carbon and nitrogen to support their biological processes. Studies have shown that bacteria require 1:20-30 proportion of nitrogen to carbon (in the form of simple sugar) during anaerobic digestion. The SWW is high in starch content but having low nitrogen content. Therefore, yeast extract was being supplemented in SWW as a nitrogen source. In this study, yeast extract gave the` positive effect, indicating that 2 g/L yeast extract is preferred to increase the hydrogen production. Past studies have revealed that 4 g/L yeast extract was needed to produce biohydrogen using *Enterobacter cloacae* [40]. The optimized condition gave a hydrogen yield of 3.1 mol H_2/mol glucose. In another investigation, it was found that yeast extract significantly affects the hydrogen production. The optimum hydrogen production was achieved when the yeast extract was 2.5 g/L [22].

3.4. Effect of temperature on the concentration of hydrogen

Temperature affects bacterial metabolic pattern, nutritional requirements and cell numbers. As a consequent, hydrogen production is also affected. *E. aerogenes* and other facultative anaerobes are mesophilic bacteria which grow in the temperature of 25-40°C [41]. However, the optimum growth temperature is reported to be 35-37°C [22, 26, 42]. In this study, temperature gave negative effect which implies that lower temperature (35°C) is preferable than the higher temperature (40°C). It was suggested that the changes in the membrane architecture is contributing to the temperature change. The membrane architecture is suspected to be associated with a hopanoid, which acts as a sterol [43]. In addition, high temperature may be resulted in rapid denaturation of protein in the enzyme which can inhibit hydrogen production [44]. This finding is consistent with the previous one which found that at the optimum temperature of 35°C, 56.5 $NmLH_2/g_{substrate}$ of hydrogen yield was produced from the municipal solid waste [45].

3.5. Effect of inoculum concentration on the concentration of hydrogen

About 5-10% of inoculum concentration was used to produce optimum hydrogen yield using single culture of *Enterobacter sp*. and *Clostridium sp*. [33, 41]. In this study, the negative effect of inoculum concentration revealed that 5% of inoculum concentration is efficient enough to produce more hydrogen rather than 15% of inoculum concentration. High inoculum concentration is not preferable since it will cause substrate competition among the bacteria. If the substrate is limited, bacteria will die and lesser hydrogen is produced, eventually. The hydrogen produced in this study is comparable to a study which supplied crude glycerol as a substrate. *E. aerogenes NRRL*

B 407 at 5% was inoculated to the crude glycerol and it produced 116.41 mmol H_2/L media [29]. In addition, the physical state of inoculum also contributes to hydrogen production. Biohydrogen production via dark fermentation is considered as growth-associated product. Hence, the hydrogen production will be optimum when the bacteria is at the mid-log phase in which the bacteria grow exponentially.

3.6. Effect of malt extract on the concentration of hydrogen

Sago starch process discharges a high amount of waste containing sugars. However, the sugars are not readily used for fermentation. Pre-treatment method is essential to reduce sugars into the fermentable sugars. Unfortunately, the pre-treatment process can cause most of the important nutrients get denatured. Hence, the improvement of biohydrogen production yield required supplementation of some macro and micro element, including malt extract. It is reported that 1% (w/v) malt extract is required to produce 3.1 mol H_2/mol glucose using *Enterobacter cloacae* IIT-BT 08 [26]. In previous investigation, supplementation of 1.5 g/L malt extract to fermentation medium had increased hydrogen yield to 5.85 mol H2/kg $COD_{reduced}$ [22]. Any increment of malt extract resulted in lower hydrogen yield due to substrate inhibition.

4. Conclusion

The optimization process of hydrogen production employs three stages of experimental design, which are screening, narrowing, and optimum search. In the first stage, Plackett-Burman design is carried out to select the important factors with respect to the production of hydrogen. In the second stage, OFAT experiment is performed to identify the optimum variable ranges by gradient method. In the last stage, the genuine optimum range can be determined by using any methods that usually assisted by the experimental software [46].

In this study, the screening process has been conducted as the first stage. Based on Plackett- Burman result, the most significant factors were yeast extract, inoculum concentration, temperature, malt extract. From this study, the highest concentration of hydrogen (454.30 µmol/L) and its yield (2.42 mmol H_2/mol glucose) were produced under the conditions: SWW concentration 100%, pH 5.5, temperature 35ºC, inoculum concentration 5%, malt extract 2 g/L, yeast extract 2 g/L, $FeSO_4.7H_2O$ 300 mg/L, $MgSO_4.7H_2O$ 100 mg/L, $CuCl_2.2H_2O$ 10 mg/L and nitrogen sparge for 3 minutes. Based on this result, the key factors affecting hydrogen production have been successfully identified.

Although the first stage of optimization has been achieved in this study, further stages to obtain final optimized condition are needed. Therefore, OFAT and response surface method are essential to be conducted. Moreover, the optimization not only can be done for the process parameters, but also the improvement of the strain. Performance of *E. aerogenes* can be enhanced by genetic modification such as mutation. Therefore, the study on the metabolic engineering of the strain is essential. A study found that the hydrogenase-3 gene cluster (*hycDEFGH*) from *E. aerogenes* has an important role in hydrogen production. It was proven that deletion of the genes, affected the integrity of hydrogenase-3, which inhibit NADH hydrogen production pathway, and no hydrogen is produced [47]. Selecting important genes and deleting inhibiting genes in hydrogen production could be one of the possible ways to improve biohydrogen production.

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