Immobile *Rhodobium Marinum* for Enhancing Hydrogen Excitation: Optimization of Environmental Factors [light intensity, pH and agitation]

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**Abstract.** Optimization of an environmental factor (pH, light intensity and agitation) for Hydrogen gas production using immobilized bacteria photosynthetic of Rhodobium marinum through facultative-photo-fermentation was studied. The experiment was conducted by using CRD (completely random design) to understand the effect of each selected parameter and their combination. The result shows that the combination of light intensity, pH and agitation were significantly affected the hydrogen production. Further data analyses gave information that light intensity and pH treatments were regulating the substrate consumption, i.e. accelerating the glucose digestion, which was, enhance the hydrogen gas production. The highest production of hydrogen gas from the experiment was in the range of 1055 - 1200 mL/L, at glucose, contain 10 g/L in the substrate. The optimizations of the photo-fermentation adaptive processes for bioproduction hydrogen gas are a necessary step to find the optimum condition of hydrogen rate and yield, in a certain period. Hydrogen gas is one of the powerful energy carriers that easy to be converting into energy form dailies such as electricity, fuel, heat or storage battery.

1. Introduction

Hydrogen seems to be looked upon as a better source of energy in comparison with other fossil-based methods due to the environmentally friendly and fulfill the new era lifestyle. Hydrogen gas generated by the fossil fuels refinery process, fixation of sunlight, wind turbine, hydropower, and hydrogen gas also found to be ably produced by fermentation series, including wastewater materials as the substrate. With the ease of the process of making the hydrogen gas, it is possible to make carrier energy in the area having specific sources (regions that are isolated or on a desert island). The process of making and burning of hydrogen does not produce substances harmful to the environment [1]. Hydrogen also can
produce the amount of energy per unit mass of the greatest of various fuels have been known which is 142 kJ/g [Hay et al, 2013]. The future of the hydrogen economy has two ways, there is hydrogen produced from domestic energy sources in a manner of affordable and environmentally benign, and its daily applications using hydrogen, i.e., fuel cell vehicles and electricity for gain market share in competition with the alternatives energies [3].

Biological hydrogen processes have involving several factors such as environmental conditions of the reactor system, pH, light intensity, temperature, agitation, medium, and microbes agent including photo-fermentative agents of Rhodobacters, Rhodobiums, Enterobacters, and dark-fermentative agent of Clostridiums [4]. Biohydrogen production is strongly influenced by the content of carbon and nitrogen substrates, hydrogen-producing bacteria and bioprocess engineering during fermentation. The environmental factors are playing a role in enzyme activity of nitrogenase and hydrogenase that response to hydrogen evolution in the microbe's cell metabolisms. In this regard, we are focused on studying the optimization of pH, light intensity and agitation to get the information of the efficient hydrogen gas bioproduction.

2. Methods

Strategies for enhancing the bioproduction hydrogen gas in this experiment are immobilizing the bacteria cells and optimization of the environmental parameters that the most influencing of the photo-fermentation process (pH level, light intensity, and agitation). The detail works are describing below.

2.1. Cultures Preparation

The photosynthetic non-sulfur purple bacterium of Rhodobium marinum was used as agent photo-fermentation. The strain was obtained from NBRC (NITE Biological Resource Center) with the collection number 100434. The stock culture was cultivated in a specific phototropic bacteria medium containing 10 g of disodium succinate, 0.3 g of yeast extract, and 10 mL of modified basal medium (MBM) diluted in 1 L of distilled water. One liter of MBM stock medium consists of 75 g of K$_2$HPO$_4$, 85 g of KH$_2$PO$_4$, 0.2 g of EDTA.2Na, 0.28 g of H$_2$BO$_3$, 0.075 g of Na$_2$MoO$_4$.2H$_2$O, 0.024 g of ZnSO$_4$.7H$_2$O, 0.21 g of MnCl$_2$, 0.004 g of Cu(NO$_3$)$_2$.3H$_2$O, 1 g of FeSO$_4$.7H$_2$O, 0.075 g of CaCl$_2$.2H$_2$O and 20 g of MgSO$_4$.7H$_2$O. The pH of the medium was adjusted to 7 before autoclaving. Nitrogen gas was spurge through a sterile medium to ensure the absence of oxygen inside the medium. The stock cultures were maintained in an incubation chamber under the following conditions: temperature of 30°C, light irradiation of 60 W/m$^2$, and gentle shaking at a speed of 120 rpm. The stock cultures were maintained for 2-3 weeks in the incubation chamber before being transferred to a new medium or cultivated in the destined media.

2.2. Cells immobilization

Immobilization was conducted using a matrix of agar with a final concentration of 1%. The agar was mixed with distilled water and stirring for 20 minutes, therefore following by sterilization using autoclave with the condition set up on 1150°C for 15 minutes. The medium is allowed to cooling down until around 30°C. R. marinum was prepared on high cell density around 10 x 10$^6$ cells or 10 levels of OD680nm (absorbance). 20% volume/volume of cell culture was added into warm agar liquid, stirring by spoon [or vortex at low speed) and let it cool down at room temperature for 2 hours. The granule used for next photo-fermentation with treatments. The immobilization procedures were following [5] method with adjustment.

2.3. Environmental Growth Factors in Batch-Photofermentation System

The R. marinum cells were placed in bottle vessels with tightly closed plugs and put in a batch glass reactor. The reactor will set in radiant of light 2000, 4000, 6000 lux; agitation of 110, 120 and 130 rpm. All parameters are combined with CCD design. The experiments are triplicate or three times
replications. The environmental treatments followed [6] with adjustment in some variables and parameters.

Table 1. Combination of the selected environmental observation parameters

| Number of Combination treatments | Light Intensity (lux) | pH | Agitation (rpm) |
|---------------------------------|----------------------|----|-----------------|
| 1                               | 2000                 | 6  | 110             |
| 2                               | 2000                 | 7  | 110             |
| 3                               | 2000                 | 8  | 110             |
| 4                               | 4000                 | 6  | 110             |
| 5                               | 4000                 | 7  | 110             |
| 6                               | 4000                 | 8  | 110             |
| 7                               | 6000                 | 6  | 110             |
| 8                               | 6000                 | 7  | 110             |
| 9                               | 6000                 | 8  | 110             |
| 10                              | 2000                 | 6  | 120             |
| 11                              | 2000                 | 7  | 120             |
| 12                              | 2000                 | 8  | 120             |
| 13                              | 4000                 | 6  | 120             |
| 14                              | 4000                 | 7  | 120             |
| 15                              | 4000                 | 8  | 120             |
| 16                              | 6000                 | 6  | 120             |
| 17                              | 6000                 | 7  | 120             |
| 18                              | 6000                 | 8  | 120             |
| 19                              | 2000                 | 6  | 130             |
| 20                              | 2000                 | 7  | 130             |
| 21                              | 2000                 | 8  | 130             |
| 22                              | 4000                 | 6  | 130             |
| 23                              | 4000                 | 7  | 130             |
| 24                              | 4000                 | 8  | 130             |
| 25                              | 6000                 | 6  | 130             |
| 26                              | 6000                 | 7  | 130             |
| 27                              | 6000                 | 8  | 130             |

2.4. Hydrogen Gas Bioproduction and the Analyses

The hydrogen produced from the treated biological materials was measured by gas chromatography (HP5890) under the following conditions: oven temperature of 80°C, detector temperature of 150°C, injector temperature of 250°C, and a carrier gas of nitrogen at a flow rate of 8 ml/min at 60 psi of pressure. The gases were observed in two steps: directly after the photo-fermentation process and after purification. For monitoring in the separated vessel, the produced hydrogen gas was collected using a "dark-impermeable bottle" secured with a tight cap and transported to the room analyses for measurement according to the method described by [7] with some modifications.
The treatment combination was based on Complete Random Design (CRD). The variable of environmental factors of agitation, light intensity and pH are evaluated and considered as parameters for statistical analyses. The analysis method followed [8] with adjustment of the variables and parameters.

3. Results and Discussion

The observation on environmental factors showed that the light intensity of 4,000 lux, pH 6 level, and agitation 120 rpm gave the highest biohydrogen production of 1055.83 – 1200 mL/L substrates. Environmental factors were suggested to regulate the substrate consumption, i.e. accelerating the glucose digestion, which enhancing the hydrogen gas production [Table 2, 3, 4].

Table 2. Hydrogen amount on treatment of initial pH 6 versus various parameters of Agitation and Light Intensity

| Time (d) | H2 Production (mL) | STDEV |
|----------|--------------------|-------|
| 0        | 0.0                | 0.0   |
| 1        | 702.3              | 5.9   |
| 2        | 609.7              | 5.1   |
| 3        | 638.7              | 15.9  |
| 4        | 545.7              | 1.5   |
| 5        | 1055.3             | 1.5   |
| 6        | 600.0              | 2.0   |
| 7        | 593.0              | 1.7   |
| 8        | 321.0              | 18.2  |
| 9        | 352.0              | 2.0   |

Note: 0: initial time, d: day/s, H2: hydrogen, STDEV: calculation of standard deviation (a measure of how widely values are dispersed from the average value (the mean)).

Table 3. Hydrogen amount on treatment of initial pH 7 versus various parameters of Agitation and Light Intensity

| Time (d) | H2 Production (mL) | STDEV |
|----------|--------------------|-------|
| 0        | 0.0                | 0.0   |
| 1        | 542.1              | 1.1   |
| 2        | 660.3              | 2.1   |
| 3        | 678.3              | 1.6   |
| 4        | 585.4              | 5.0   |
| 5        | 517.9              | 15.8  |
| 6        | 1299.0             | 1667.1|
| 7        | 800.7              | 0.6   |
Table 4. Hydrogen amount on treatment of initial pH 8 versus various parameters of Agitation and Light Intensity

| Time (d) | H2 Production (mL) | STDEV |
|----------|---------------------|-------|
| 0        | 0.0                 | 0.0   |
| 1        | 519.8               | 1.3   |
| 2        | 463.7               | 2.0   |
| 3        | 541.4               | 1.5   |
| 4        | 525.7               | 1.1   |
| 5        | 405.0               | 4.3   |
| 6        | 484.8               | 2.3   |
| 7        | 660.6               | 1.2   |
| 8        | 286.5               | 1.8   |
| 9        | 232.0               | 1.0   |

Note: 0: initial time, d: day/s, H2: hydrogen, STDEV: calculation of standard deviation (a measure of how widely values are dispersed from the average value (the mean)).

Those tables are revealing pH 6 better than the other pH level for enhancing the hydrogen gas production, which might be due to the bacteria's non-sulfur purple bacteria including Rhodobium, which has a preference habitat at low-level pH. PH level is one of the parameters that have a strong influence on the hydrogen evolution in cell metabolisms, due to strongly bond to nitrogenase and hydrogenase enzyme activities [9]. The initial pH level of 6 seemed to give a stable condition on the batch-photofermentative system with 1% glucose substrate, 1% agar immobilization matrix and cell density around OD$_{680nm}$ of 1.0. The other observation showed that light intensity also affected the hydrogen bioproduction. The radiant of 4,000 lux gives the highest production of the hydrogen gas [Table 5].

Table 5. Hydrogen production on various light intensity treatment

| Combination Treatment On Light Intensity Treatment (Lux) | H2 Production mL | STDEV |
|----------------------------------------------------------|------------------|-------|
| 1                                                        | 656.68           | 3.22  |
| 2                                                        | 542.72           | 1.55  |
| 3                                                        | 509.47           | 9.22  |
| 4                                                        | 603.66           | 0.57  |
The light intensity suggested influences the hydrogen gas excitation in cell metabolism since the *R. marinum* is photosynthetic microbes. The radiant light is an important growth factor for doing photosynthesis and also excitation of the hydrogen gas from the cells [10]. Observation on agitation treatments of 110, 120, and 130 rpm showed in general 110 rpm of agitation have higher hydrogen gas production compare to other strangeness of agitation (120 and 130 rpm) (Figure 3). However, some points at 120 and 130 rpm agitation conditions give a slightly higher than average hydrogen production gas on 110 treatments condition.

Table 6. Hydrogen production on various agitation

| Combination Treatment On Agitation Treatment (RPM) | H2 Production (mL) | STDEV |
|--------------------------------------------------|-------------------|-------|
| 1                                                | 657.35            | 3.06  |
| 2                                                | 544.05            | 0.92  |

Note: H2: hydrogen, STDEV: calculation of standard deviation (a measure of how widely values are dispersed from the average value (the mean)). Number 1-9 was subjected to 2,000-lux light intensity; 10-18 was subjected to 4,000 light intensity; and 19-27 was subjected to 6,000-lux light intensity. The light intensities were performed by fluorescent lamps.
3  509.47  9.22
4  602.99  2.64
5  664.27  3.00
6  462.39  2.51
7  623.14  1.50
8  673.87  3.45
9  546.36  7.73
10 554.03  6.51
11 585.35  5.04
12 526.36  7.09
13 1950.33 50.41
14 537.56 15.96
15 404.86  4.22
16 602.69  2.52
17 386.70  4.11
18 484.77  6.37
19 593.97  4.59
20 803.72  5.47
21 508.05  67.88
22 498.37  7.61
23 383.65  6.24
24 419.68  17.05
25 359.24  6.77
26 349.72  5.55
27 244.73  5.10

Note: H2: hydrogen, STDEV: calculation of standard deviation (a measure of how widely values are dispersed from the average value (the mean)). Number 1-9 was agitated on 110 rpm; 10-18 was agitated on 120 rpm; and 19-27 was agitated on 130 rpm. The agitation was performed by shaker rotary.

The agitation is important factor for nutrition distribution, accelerated the enzymes activities and ion excitation in the cultures [11].

Synchronized data showed that light intensity of 2,000 lux give slightly higher hydrogen production compare to other factors (Table 7). On the other hand, the highest hydrogen production was achieved by treatment of 4,000 light intensity.

Table 7. Interpretation of Data-cross from the experiment

| Number of Combination Treatment | Light Intensity (lux) | pH | Agitation (rpm) | Average Hydrogen Gas Production (mL) | Average Usage Glucose concentration (ppm) |
|-------------------------------|-----------------------|----|-----------------|-------------------------------------|------------------------------------------|
| 13                            | 4000                  | 6  | 120             | 1055.83                             | 6794.35                                  |
Further observation is needed in order to find information of hydrogen evolution in typically batch-photo-fermentative system with immobilized microbes.

4. Conclusion
The highest hydrogen production was achieved by treatment of 4,000-lux light intensity, adjustment of 6 levels of initial pH and 120 rpm agitation. The condition of the microbe agent was immobilized by 1% agar with a yield around 1055 mLH₂gas/1L substrate. Further observation is needed to find the optimum condition for hydrogen production using a typical photo-fermentative bioreactor.

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6. References
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