Growth Study and Biological Hydrogen Production by novel strain *Bacillus paramycoides*

Eldon Chung Han Chua¹, Siaw Khur Wee¹, Jibrail Kansedo¹, Sie Yon Lau¹, King Hann Lim², Sharul Sham Dol³, Anuj Nishanth Lipton⁴.

¹Department of Chemical Engineering, Faculty of Engineering and Science, Curtin University Malaysia, CDT 250, 98009 Miri, Sarawak, Malaysia.
²Department of Electrical & Computer Engineering, Faculty of Engineering and Science, Curtin University Malaysia, CDT 250, 98009 Miri, Sarawak, Malaysia.
³Department of Mechanical Engineering, Abu Dhabi University, 59911, Zayed City, Abu Dhabi, United Arab Emirates.
⁴Curtin Malaysia Research Institute, Curtin University Malaysia, CDT 250, 98009 Miri, Sarawak, Malaysia.

Corresponding author: eldonchuach@postgrad.curtin.edu.my

Abstract

Industrial revolution has created high dependent on fossil fuels for energy creation. However, combustion of fossil fuels has created excessive amount of greenhouse gases, hence led to climate change. Thus, renewable energy has been proposed to alleviate the environmental pollution issues around the globe. One of the promising renewable energies is green hydrogen energy. Commercialized technologies such as electrolysis and thermochemical reaction are utilized to form hydrogen energy. Nonetheless, these processes require high energy and yet producing greenhouse gases that harm the environment. In this study, biodegradation process to produce hydrogen energy has been explored. To our knowledge, *Bacillus paramycoides* strain has not yet been investigated for biological hydrogen evolution. Therefore, in this paper, the ability of *Bacillus paramycoides* to produce biological hydrogen has been studied. The rod-shaped and gram-positive *Bacillus paramycoides* was identified under scanning electron microscope and gram staining procedure. Furthermore, biological hydrogen generation by *Bacillus sp.* was experimented for 96 hours. The result shows that 4668 ± 120 ppm cumulative
hydrogen gas was generated through dark fermentation process. For *Bacillus sp.* growth study, lag, log, and stationary phase have been achieved in 96 hours. In a summary, metabolic engineering to degrade abundant biomass wastes is a sustainable pathway to produce hydrogen energy, simultaneously resolve waste management issue around the globe.

**Keywords:** Green energy, Microbes degradation, Biological hydrogen production, Dark Fermentation

### 1. Introduction

Energy is essential to fuel up our daily processes. Oil crisis in 1970s indicated that fossil fuel is not sustainable for future energy creation. Industrial revolution has created high dependent on fossil fuels for energy production. However, combustion of oil and gas produced excessive amount of greenhouse gases, hence led to climate change [1], [2]. Therefore, energy transition is crucial to reduce fossil fuels for energy creation. Renewable energy has been proposed to resolve environmental pollution issues. One of the renewable energies that receive much attention is the hydrogen energy. Properties such as high calorific value (140 kJ/g), carbon neutral, and environmentally friendly could make hydrogen as an important element in the energy sector [3], [4]. Nonetheless to create hydrogen energy, further process is required to isolate hydrogen from molecules such as hydrocarbons, water, and acids [5]. Commercialized electrolysis and thermochemical processes are utilized to create hydrogen element. These processes require fossil fuels or high energy to operate, creating greenhouse gases which making it unsustainable for the future [6]. To overcome these issues, organic substrate degradation by microorganisms could be a better alternative to create green hydrogen energy. The biorefinery pathway is able to degrade biomass into valuable biochemicals by-product, or even biofuel [7]. For instance, energy-efficient and environmentally friendly dark fermentation technology is proposed to create green hydrogen energy [8].
Anaerobic microorganisms are utilized in dark fermentation to transform organic substrates into hydrogen energy. For instance, Bacillus sp., Enterobacter sp., and Clostridium sp. are the most common hydrogen producing strains [9]. Furthermore, agricultural wastes, food wastes, and wastewater can be utilized and treated through dark fermentation process [10]. The phenomenon of dark fermentation can be described as:

\[
C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \tag{2.1}
\]

\[
C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COOH + 4H_2 + 2CO_2 \tag{2.2}
\]

\[
3C_6H_{12}O_6 \rightarrow 4CH_3CH_2COOH + 2H_2O + 2CO_2 + 2CH_3COOH \tag{2.3}
\]

Although many efforts have been made to produce biological hydrogen from fermentation technology, the main limitation is still the low hydrogen yield from fermentative bacteria. Besides, different microorganism strains possess different potential for hydrogen production. Therefore, in the view of sustainable biofuel generation, development of biological hydrogen generation from fermentation demands further exploration of new hydrogen-producing strains. This research work aims to explore the ability of novel strain Bacillus paramycoides to produce biological hydrogen at controlled operating condition.

2.0 Materials and Methodology

For activation process, Bacillus sp. was grown and activated in borosilicate conical flask for 24 hours prior to experimental study. There were 10g of glucose, 3g of peptone, 1g of yeast extract, 2.8g of K2HPO4, 3.9g of KH2PO4, 0.2g of MgSO47H2O, 0.1g of NaCl, 0.01g of CaCl26H2O, 0.05 g of FeSO47H2O, 0.2g of L-cysteine, and 1mL microelements in 1L of culturing and activation media. The initial pH value for both activation and cultivating media were recorded as 6.70. Besides, the microelements utilized was 1L solution that contain 0.07g of ZnCl2, 0.1g of MnCl24H2O, 0.06g of H3BO3, 0.2g of CoCl26H2O, 0.02g of CuCl22H2O, 0.02g of NiCl26H2O, and 0.04g of NaMoO42H2O. Before the experimental work, conical flasks which contain activation and cultivation media were autoclaved at 121°C for 20 minutes to ensure the sterile condition. For carbon source, it
was autoclaved separately at 110°C for 20 minutes before added into the activation and cultivation media. Moreover, the activation and culture mediums were flushed with argon gas for 15 minutes to remove excess oxygen. This process is to ensure the anaerobic condition for biological hydrogen fermentation by Bacillus sp.. The activation and cultivation process were carried out in the refrigerated static incubator controlled at 33°C. Additionally, Thermo Fisher ESEM Quattro S was used to study the microorganism’s colony structure and morphology. Gram staining procedure was carried out to identify the gram stain characteristic of Bacillus sp. (Figure 2.1). Lastly, the DNA was isolated and identified through 16S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR) for identification purpose.

Figure 2.1: Gram staining procedure [11].

For gram staining procedure, cells were air-dried and heat-fixed on a microscope glass slide. The heat-fixed cells were flooded with 60 seconds crystal violet staining reagent. In the following step, glass slide was washed with gentle stream of tap water for 2 seconds followed by gram's iodine flooding for 60 seconds. Furthermore, the glass slide was washed with ethyl alcohol for 5 to 10 seconds to remove the iodine reagent. Lastly, safranin which acted as the counterstain was flooded on the glass slide for roughly 45 seconds, followed by flushing indirect stream of tap water until no colour appeared in the effluent. The result was observed under Optical Microscope Eclipse LV1100ND (NIKON).

For inoculating preparation and analytical process, the experimental work was performed in Class II Biological Safety Cabinet EFD-5AB (ESCO SCIENTIFIC) to avoid contamination. The growth behaviour and biological hydrogen production from Bacillus sp. were analysed every 4 hours up to 96 hours. The biological hydrogen production was
analysed by a portable hydrogen detector (ATO). For bacteria growth study, solution sample was analysed every 4 hours by UV-VIS Spectrometer Lambda 365 (PERKIN ELMER), with OD 600nm for *Bacillus sp.*. Furthermore, the optical density from UV spectra reading was converted to cell number where 1 OD of 600nm is $1.0 = 8 \times 10^8$ cells. The results are demonstrated in the following section.

### 3.0 Results and Discussion

#### 3.1 Bacteria Identification

The cell was observed under scanning electron microscope (Figure 3.1). It was identified as a rod-shaped bacterium with cell length of 1.8-2.2 µm, and cell width of 0.8-1.2 µm. This fits one of the characteristics of *Bacillus sp.*, where it is a rod-shaped growing pattern bacteria [12].

![Figure 3.1: Bacillus sp. under scanning electron microscope (SEM).](image)

In addition, gram staining procedure was performed to identify whether the cells are gram-positive or gram-negative microorganisms. The result is illustrated in Figure 3.2:
The difference between gram-positive and gram-negative bacteria is gram-positive bacteria has thick layers of peptidoglycan in the cell walls. For gram-negative bacteria, it has a thin layer of peptidoglycan in the cell walls [13]. Besides, to differentiate gram-positive and gram-negative bacteria, gram staining procedure can be employed. The organisms that retain the primary colour and appear purple under a microscope is gram-positive organisms, whereas organisms that appear red under a microscope are gram-negative organisms [11]. According to literature, Bacillus sp. are gram-positive rods microorganisms [14]. Thus, the gram staining procedure has proved the identity of the cell as gram-positive organisms and showing purple colour staining under a microscope. Moreover, to reconfirm the strain utilized, the DNA was isolated and identified under the 16S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR). The phylogenetic tree (Figure 3.3) classifies the dark fermentative bacteria as Bacillus paramycoides.
Figure 3.2: Bacillus sp. (A) before and (B) after gram staining.

The difference between gram-positive and gram-negative bacteria is that gram-positive bacteria have thick layers of peptidoglycan in the cell walls, whereas for gram-negative bacteria, it has a thin layer of peptidoglycan in the cell walls [13]. Besides, to differentiate gram-positive and gram-negative bacteria, gram staining procedure can be employed. The organisms that retain the primary color and appear purple under a microscope is gram-positive organisms, whereas organisms that appear red under a microscope are gram-negative organisms [11]. According to literature, Bacillus sp. are gram-positive rods microorganisms [14]. Thus, the gram staining procedure has proven the identity of the cell as gram-positive organisms and showing purple colour staining under a microscope.

Moreover, to reconfirm the strain utilized, the DNA was isolated and identified under the 16S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR). The phylogenetic tree (Figure 3.3) classifies the dark fermentative bacteria as Bacillus paramycoides.

![Figure 3.3: Phylogenetic tree for DNA isolate.](image)

After identifying the cell as gram-positive, rod-shaped *Bacillus paramycoides*, a respective growth behaviour study and the hydrogen producing ability was examined. The results are included in section 3.2 and 3.3.

### 3.2 Growth Study of Dark Fermentative Bacteria

Figure 3.4 illustrated the growth behaviour of *Bacillus sp.* during dark fermentation process. The fermentation liquid samples were collected and analysed every 4 hours up to 96 hours.
From Figure 3.4, it can be observed that the lag phase of cell occurred during the first 20 hours of fermentation. This may be due to the Bacillus sp. adaption into a new growing environment. Moreover, log phase behaviour happened after the 20th hours where maximum doubling rate appeared for the microbes. The log behaviour lasted up to 44 hours. Lastly, stationary phase took place after 48 hours until the end of fermentation time where there is no net increase in microbe’s numbers. Simultaneously, the hydrogen production of Bacillus sp. was analysed during the growth behaviour study.

4.2 Biological Hydrogen Production by Dark Fermentation

Figure 3.5 illustrated the biological hydrogen produced by Bacillus sp. during dark fermentation process. The biological hydrogen production was analysed every 4 hours up to 96 hours.
Figure 3.4: Growth curve study of Bacillus sp. during dark fermentation.

From Figure 3.4, it can be observed that the lag phase of cell occurred during the first 20 hours of fermentation. This may be due to the Bacillus sp. adaption into a new growing environment. Moreover, log phase behaviour happened after the 20th hours where maximum doubling rate appeared for the microbes. The log behaviour lasted up to 44 hours. Lastly, stationary phase took place after 48 hours until the end of fermentation time where there is no net increase in microbe’s numbers.

Simultaneously, the hydrogen production of Bacillus sp. was analysed during the growth behaviour study.

4.2 Biological Hydrogen Production by Dark Fermentation

Figure 3.5 illustrated the biological hydrogen produced by Bacillus sp. during dark fermentation process. The biological hydrogen production was analysed every 4 hours up to 96 hours.

Rapid biological hydrogen production occurred during the first 24 hours of fermentation. Biological hydrogen generation by Bacillus sp. increased proportionally from 0 to 24th hours, then reduced significantly. Starting from the 48th hours, a stable hydrogen production was achieved up to 96 hours. Cumulative biological hydrogen produced was 4668 ± 120 ppm. From Figure 3.4 and Figure 3.5, rapid biological hydrogen production rate appeared during the first 20 hours of fermentation, which is the lag phase of Bacillus sp. growth. This phenomenon can be explained by the density-dependent communication system of microorganisms which also known as quorum sensing [15].

Quorum sensing involves cell–cell communication process which responsible for production, detection, and response towards extracellular signalling molecules named autoinducers. In a result, Bacillus sp. may send autoinducer signals between each other to improve their microbial concentration during lag phase. In addition, quorum sensing regulates enzyme production for microbial growth purpose [16]. Thus, the high-rate communication between cells may increase the enzyme production which in turn increasing the biological hydrogen formation. Lastly, when Bacillus sp. achieved the stationary growth phase, decreasing rate of density-dependent communication system may reduce the rate of enzyme production. This may decrease the biological hydrogen
formation and resulted a stable hydrogen production rate. Therefore, community of inter-bacteria species has significant impact on biological hydrogen production rate in fermentation process.

The experimental results found that \textit{Bacillus paramycoides} could generate biological hydrogen through dark fermentation process by utilizing glucose as the feedstock. In a comparison with other studies, Ziara et al. (2019) used lactate wastewater as the substrate for dark fermentation technology. Temperature and initial pH are the affecting variable for biological hydrogen yield. The research work determined that mesophilic temperature of 35℃ - 45℃ and initial pH of 6.5 - 8.5 were the optimized conditions for biological hydrogen production. The optimized biological hydrogen yield was 0.91 mol \textit{H}_2 / mol lactate wastewater with operating conditions of 45℃ and 7.5 pH condition [17]. Furthermore, Li et al. (2020) investigated dark fermentation with different salinity and pH of swine wastewater. The study showed that pH 6 and 1.5% salinity of swine wastewater was optimum for dark fermentative hydrogen formation. The experimental work also studied that biological hydrogen yield was affected by soluble chemical oxygen demand during alkaline condition and 3-3.5% high salinity solution [18]. Additionally, micronutrients such as Zn, Mn, Ca, Co, Ni, Fe, Cu have significant impact towards the growth and hydrogen production of dark fermentative microbes. Enzymes such as nitrogenase and hydrogenase produced from fermentative microbes may be enhanced or inhibited by the addition of microelements [19]–[24]. Therefore, \textit{Bacillus paramycoides} could be further experimented with various operating conditions to enhance its ability for hydrogen production. Different type of biomass wastes can also be experimented with \textit{Bacillus paramycoides} dark fermentation for useful bioproducts formation.

\textbf{5.0 Conclusion}

After the cell identification process, the DNA was proved to be gram-positive, rod-shaped organisms which show purple colour staining under a microscope. The cell was identified as \textit{Bacillus paramycoides} (MCCC 1A04098). For cell growth behaviour study, lag phase achieved was 20 hours, followed by 28 hours of exponential phase and 48 hours of
stationary phase. Moreover, cumulative biological hydrogen produced was 4668 ± 120 ppm from *Bacillus sp.* It was found that cell number has significant impact on biological hydrogen production rate due to the density-dependent communication system. In a nutshell, the cell identification and basic hydrogen fermentation trial found that *Bacillus paramycoides* are feasible to produce hydrogen. This article provides new evidence for the potential of hydrogen production from fermentation process. Further experimental work on investigating the effect of various operating conditions towards *Bacillus sp.* hydrogen fermentation is crucial. In the meantime, exploring novel strains, genetic engineering, reactor system optimization, and substrates processing methods can also be explored for hydrogen fermentation commercialization. This is to ensure sufficient hydrogen energy to be generated to support the world energy crisis.

**Acknowledgements**

This work was supported by Curtin University Malaysia under the Fundamental Research Grant Scheme (FRGS/1/2019/TK10/CURTIN/02/1).

**References**


