NOVEL STRAINS *BACILLUS PARAMYCOIDES* & *CEREIBACTER AZOTOFORMANS* FOR BIOLOGICAL HYDROGEN PRODUCTION: A PARAMETRIC STUDY

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Abstract

Biodegradation is a promising method for producing hydrogen, a clean and renewable energy source, from organic waste materials. This process involves the use of microorganisms, such as bacteria, to break down organic matter into simpler compounds and produce hydrogen as a biological by-product. The biodegradation of waste materials, such as agricultural residues, food waste, and wastewater, not only produces hydrogen but also provides an eco-friendly solution for waste management. Several factors affect the efficiency of hydrogen production through biodegradation, including the type and concentration of organic matter, the type of micronutrients, pH, and the type and inoculum size of microorganisms used. Therefore, optimization of these factors is crucial for maximizing hydrogen production. In this project, novel strains *Bacillus paramycoides* and *Cereibacter azotoformans* were explored for dark and photo fermentation processes to produce biological hydrogen. Various operating parameters were experimented with, including the concentration of glucose and xylose, types of metal ions, and bacterial inoculum sizes for hydrogen generation. The study shows that these operating conditions have a significant effect on the biological hydrogen yield from the fermentation process. In a summary, biodegradation-based hydrogen production offers a promising avenue for producing clean and renewable energy while simultaneously addressing the pressing issue of waste management.

Keywords: *anaerobic biodegradation, biological hydrogen, dark fermentation, photo fermentation, parametric study*

1. Introduction

In this burgeoning epoch of science and technology, the demand for renewable and sustainable energy sources has increased significantly due to the depletion of fossil fuels and the growing concerns over environmental pollution [1]. The over-reliance of the world on a vast array of fossil fuels has resulted in the production of a large quantity of greenhouse gases (GHG). These GHG are generated by the combustion of fossil fuels and have caused climate change and global warming, resulting in a rise in sea level [2]. Hydrogen is considered as a clean and versatile energy carrier. It has drawn much attention as a potential solution for long term storage of renewable energy [3]. Currently, most of the hydrogen energy are derived from fossil fuels, which not only contribute to greenhouse gas emissions but also raise concerns over the limited availability of these resources. In fact, it is estimated that hydrogen derivation from fossil fuels accounts for about 830 million tons of carbon dioxide annually (MtCO_2/ year), which is a significant contributor for climate change [4]. In a result, there is a growing interest in developing alternative methods for hydrogen generation that rely on renewable and sustainable resources.

Biodegradation is one of the promising approaches to produce renewable hydrogen energy. It involves the conversion of organic matter into hydrogen gas by microorganisms under anaerobic condition. Abundant lignocellulosic and biomass wastes such as palm oil mill effluent, winery wastewater, paper waste, post-harvest agricultural waste, and woody biomass have contributed significantly towards the economic viability of sustainable biopathway hydrogen formation [5]–[7]. 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. Despite the progress made in identifying novel microbial strains for hydrogen production through biodegradation, there remains a need to screen a greater number of strains with higher hydrogen-producing capabilities to ensure the viability of future commercialization processes [8]. In this regard, the ability of novel strains dark fermentative *Bacillus paramycoides* and photo fermentative *Cereibacter azotoformans* to produce biological hydrogen have been explored.

The biological hydrogen yield from biodegradation process is influenced by various factors, including type and substrate composition, microbial inoculum size, and micronutrients availability. In an experimental research study, various types of sugar were utilized for biodegradation process to produce hydrogen molecules. The organic substrates include sucrose, glucose, lactose, fructose, and xylose. The comparison study found that hydrogen production was the highest when xylose was utilized as the substrate for fermentation. The hydrogen yield were 240mL H_2 /g sucrose, 270 mL H_2 /g glucose, 150 mL H_2 /g lactose, 140 mL H_2 /g fructose, and 280 mL H_2 /g xylose with different carbon sources at 10 g/L concentration [9]. Another research project discovered that the initial concentration of cells has a notable impact on the biological hydrogen production from the fermentation process. The study examined initial yeast concentrations of 5% and 9% to determine their effect on biological hydrogen production. The results indicated that the 5% inoculum size generated a higher yield of biological hydrogen compared to the 9% inoculum size, with hydrogen yields of 52 μmol and 32 μmol, respectively [10]. Moreover, Zhang et al. (2021) revealed that the

introduction of cobalt ferrate nanoparticles had improved the hydrogen evolution from dark fermentation. The mesophilic *Clostridium* anaerobic sludge was utilized in their dark fermentation work. The introduction of 0.4g/L of $CoFe_2O_4$ released Fe^{3+} and Co^{3+} into the fermentation broth upon corrosion, hence stimulated the biological hydrogen evolution by roughly 32%. The hydrogen yield was 206.03mL H_2 /g glucose. The authors also reported that increasing in $CoFe_2O_4$ concentration may inhibit enzyme activity to produce biological hydrogen [11].

Optimizing the fermentation process to produce higher hydrogen yields is crucial, and understanding the impact of various factors is essential in achieving this objective. In this study, novel strains were evaluated for their hydrogen-producing capabilities using xylose and glucose as the substrate in a series of batch fermentations. The hydrogen yield and production rate were measured under different substrate concentrations ranging from 2.5 to 20 g/L. Additionally, the effect of microbial inoculum size on hydrogen production was investigated by varying the inoculum size from 2 to 10 g/L. Various types of metal ions were also experimented with to investigate the effect of micronutrients on biological hydrogen yield from dark and photo fermentation processes. The findings from this study provide valuable insights into the factors that impact the production of biological hydrogen by novel microbial strains, and can be used to optimize the fermentation process to achieve higher hydrogen yields, leading to significant enhancement in hydrogen production efficiency and stability. This research is significant as it contributes to the development of sustainable and environmentally friendly methods for energy production, which is crucial in mitigating the impact of climate change. Moreover, the metabolic pathways of the strains also provide a foundation for further research and development in this field.

2. Materials and Methods

DSMZ provided freeze-dried cells that were activated in Pyrex borosilicate conical flasks for 48 hours before the start of the experiment. To cultivate the photo fermentative microbe, a nutrient broth solution was used, containing 8g nutrient broth in 1L of Milli-Q water. In contrast, the dark fermentative microbe was grown in a culture media that included 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, 0.05g of FeSO47H2O, 0.2g of L-cysteine, and 1mL of microelements in 1L of solution. The microelements solution (1L) included 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 [12]. Prior to each experiment, conical flasks and cultivating media were autoclaved (HV-110 Hirayama) at 121°C for 20 minutes. Carbon sources were autoclaved separately at 110°C for 20 minutes before being added to the cultivating media.

To conduct the parametric study on dark and photo fermentation, hydrogen generation was carried out in Duran borosilicate test tubes with a working volume of 30mL. The fermentation was performed in the respective growth medium, but with different types and concentrations of carbon source, various types of metal ions, and different sizes of cell inoculum. The test tubes and cultivating media were autoclaved at 121°C. Xylose and

glucose were autoclaved separately at 110°C for 20 minutes. The cells were activated in the growth medium, while the inoculum was obtained during the bacteria log phase at the 24th hour of activation. Additionally, the parametric study was repeated at least three times, and the average results were used to plot the graph in the Results and Discussion section. To create anaerobic conditions for cell activation and the parametric study, the conical flask and test tubes were flushed with argon gas for 15 minutes to remove oxygen and then closed with a rubber stopper. Furthermore, bacteria culturing was performed in a closed desiccator with lighted candles to remove excess oxygen from the environment. For light fermentation by *Cereibacter azotoformans*, it was constantly supplied with illumination by a 300W Ultra-Vitalux lamp. Figure 2.1 illustrates the experimental setup for cell cultivation and quantification of biological hydrogen yield.

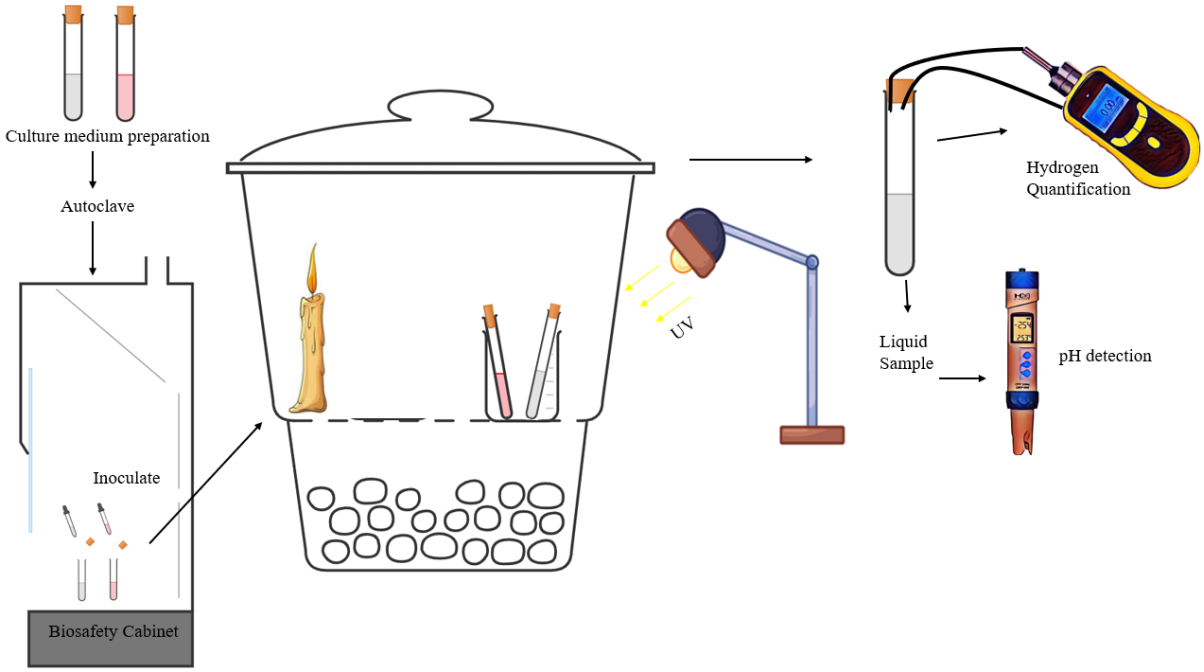

Figure 2.1: Experimental set-up for biological hydrogen fermentation.

The analytical approach is being utilized to oversee the pH fluctuations in the fermentation medium and to determine the quantity of biological hydrogen generated through the dark and photo fermentation procedures. To analyse the pH, 1 mL samples of the solution were collected periodically every 24 hours and subjected to analysis using a portable pH meter (Yieryi). However, for ease of discussion, only the final pH reading is displayed on the graphs in the Results and Discussion section to facilitate comparison among different operational conditions. Additionally, a portable hydrogen gas detector (ATO) fitted with an electrochemical detector was employed to measure the biological hydrogen concentration produced by both dark and photo fermentation processes. To measure the gas produced from the fermentation process, the inlet and outlet of the hydrogen detector were connected to the conical flask, forming a loop to detect the real-time concentration in the conical flask. The hydrogen detector was operated at a constant pump rate of 75 ml/min. After recording the

hydrogen reading from the detector, the outlet was unplugged, and the detector continued to pump to flush away the hydrogen gas in the conical flask, thus resetting the hydrogen gas concentration to zero. The process was repeated every 24 hours to measure the hydrogen gas produced periodically, and the hydrogen concentration was added for cumulative calculation after three days. Furthermore, to prevent contamination, the inoculation, liquid sample collection, and hydrogen quantification procedures were conducted in a class II biological safety cabinet (Esco Scientific).

3. Results and Discussion

The strains were identified by means of a scanning electron microscope (SEM) and 16S rRNA gene sequencing. Their relation to other known strains was determined, and DNA was isolated and identified through the use of the 16S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR). The 16S rRNA gene from bacterial isolates' gDNA was PCR-amplified using primer 785F and 907R after extracting the total DNA. The amplified product was documented after being run on a 0.6% agarose gel, and it showed distinct DNA bands with an approximate size of 1500 base pairs. After running the PCR amplified products on a 1% agarose gel, Lane M was used to represent the DNA ladder (DNA Ladder Mix 250 to 10000 base pair, catalogue number BIO-5140). The size of markers with high intensity was shown. This analysis shows that there was specific amplification of the 16S rRNA gene. Sequence analysis of both the forward and reverse primers was performed and the results were assembled into one full-length sequence after editing.

(A) (B)

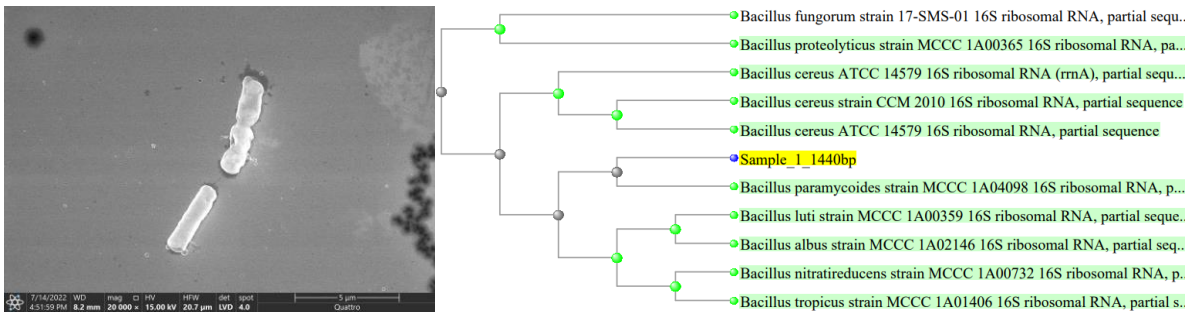

Figure 3.1: Representative image of (A) *Bacillus paramycoides* under SEM at 20000X after growth on activation medium, along with its (B) phylogenetic tree.

The bacterium was identified as having a rod shape, with a cell length ranging from 1.8-2.2 µm and a cell width ranging from 0.8-1.2 µm. Analysis of the 16S rRNA gene sequence revealed that the cell shared more than 99.93% similarity with *Bacillus paramycoides* (MCCC 1A04098). Figure 3.2 displays *Cereibacter azotoformans* under SEM, along with its phylogenetic tree.

(A) (B)

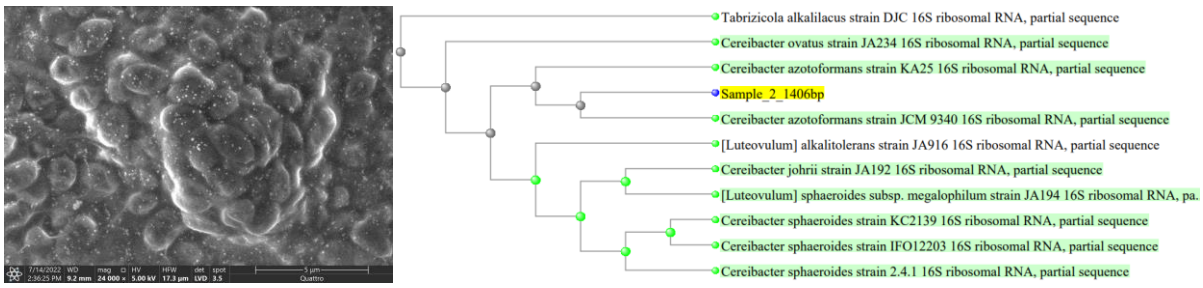

Figure 3.2: Representative image of (A) *Cereibacter azotoformans* under SEM at 24000X after growth on activation medium, along with its (B) phylogenetic tree.

The cell was identified as an ovoid bacterium with a cell length ranging from 1.5-2 µm and a cell width ranging from 1.2-1.5 µm. Analysis of the 16S rRNA gene sequence revealed that the cell shared more than 100% similarity with *Cereibacter azotoformans* (JCM 9340). After identifying the strains, the biological hydrogen production by dark fermentative *Bacillus sp.* was studied based on various concentrations of xylose and glucose, ranging from 0 to 20 g/L of carbon source.

Biological hydrogen production from (A) xylose and (B) glucose by dark fermentation

(A) (B)

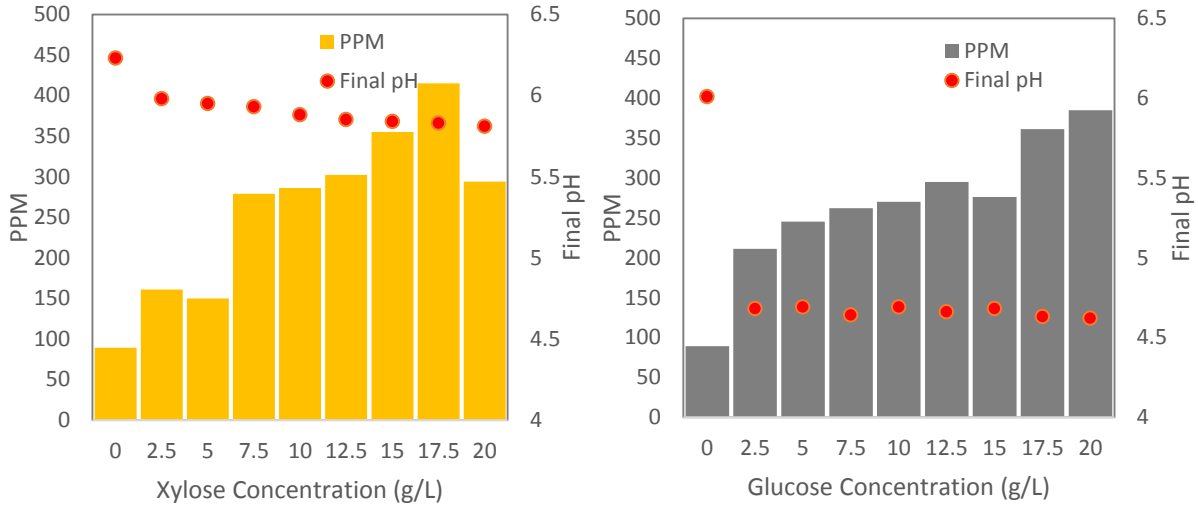

Figure 3.3: Cumulative biological hydrogen production by *Bacillus sp.* based on various concentration of xylose and glucose.

The main components of plant biomass are cellulose, hemicellulose, and lignin. After pre-treatment and saccharification of plant biomass, the most abundant monosaccharides in biomass hydrolysates are glucose and xylose [13], [14]. Therefore, glucose and xylose were used for biological hydrogen fermentation. Figure 3.3 shows the biological hydrogen production by dark fermentative Bacillus sp. over a period of three days. For xylose, the

optimal concentration for biological hydrogen production by *Bacillus sp.* was 17.5 g/L with a cumulative hydrogen production of 415ppm. For glucose, the optimal concentration for biological hydrogen production was 20 g/L, with 385ppm of biological hydrogen accumulated. The graph demonstrates that a higher carbon source results in a higher biological hydrogen formation. A research report demonstrated that proper carbon source addition can alleviate the inhibition of metabolites and feedstocks, improving bacterial metabolism and increasing by-product productivity [15]. In our case, the optimal biological hydrogen accumulation for xylose addition was 415ppm, compared to 89ppm for 0 g/L of xylose concentration, representing a 366% enhancement in hydrogen accumulation. Additionally, 20 g/L glucose concentration showed approximately 432% improvement in biological hydrogen accumulation compared to 0 g/L of glucose. However, the further addition of carbon sources may inhibit by-product formation in the fermentation process. As illustrated in Figure 3.3, 20 g/L xylose concentration resulted in only 294ppm of biological hydrogen. Research studies have shown that with higher feedstock concentration, bacteria metabolism may shift from hydrogen fermentation to acid fermentation, which increases toxicity, causes pH reduction, reduces enzyme activity, and affects biological hydrogen yield [16], [17]. Furthermore, Figure 3.3 shows that a higher hydrogen yield was obtained when xylose was used as the substrate. This may be due to the higher concentration of organic acids formed during glucose fermentation. It has been reported that the accumulation of acetate and butyric acids is higher in glucose fermentation than in xylose fermentation [9]. The initial pH was recorded as 6.7 before fermentation. The final pH for glucose fermentation was recorded as 4.62 to 4.69, whereas the final pH for xylose fermentation was 5.81 to 5.98. Jarunglumlert, Prommuak, Putmai, and Pavasant (2018) found that biological hydrogen production from fermentation is optimal at a pH of 5 to 6, facilitating the transportation of nutrition and cells by maintaining surface charge on bacteria cell membranes [18]. Therefore, the hydrogen-producing enzyme by *Bacillus sp.* may have the highest activity when xylose is utilized as the feedstock, with a suitable pH resulting in hydrogen fermentation.

Parametric study of photo fermentation by *Cereibacter sp.* was performed based on various xylose and glucose concentration. The results are illustrated in Figure 3.4:

Biological hydrogen production from (A) xylose and (B) glucose by photo fermentation

(A) (B)

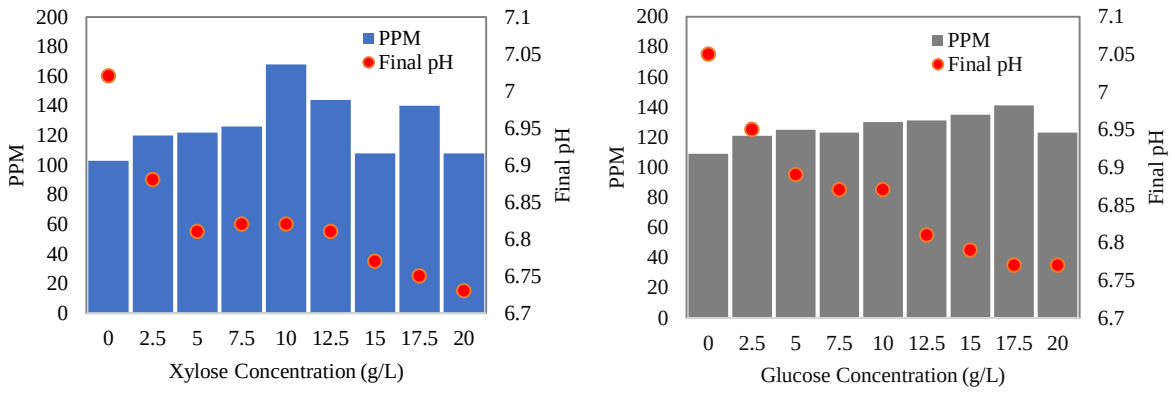

Figure 3.4: Cumulative hydrogen production by *Cereibacter sp.* based on various concentration of xylose and glucose.

Figure 3.4 illustrates that the optimal biological hydrogen accumulation by *Cereibacter sp.* occurred when the substrate consisted of 10 g/L of xylose and 17.5 g/L of glucose. The accumulated biological hydrogen for xylose and glucose was 168 ppm and 141 ppm, respectively. Yang and Wang (2018) reported that optimizing the nutrition provided to microbes can balance the interaction with substrates, improve microbial metabolism, and increase biological hydrogen productivity [15]. In addition, Figure 3.4 demonstrates that the higher the concentration of carbon sources, the higher the accumulation of biological hydrogen after fermentation. Nevertheless, further increases in carbon source concentration result in decreasing biological hydrogen production. For xylose concentration above 10 g/L and glucose concentration above 17.5 g/L, biological hydrogen production inhibition can be observed, resulting in reduced hydrogen accumulation. Studies have revealed that the higher the substrate concentration, the higher the accumulation of VFAs after biological fermentation, which can lead to a more acidic fermentation medium and inhibit nitrogenase activity to form biological hydrogen [19][20].

Besides, the pH of the photo fermentation medium was initially recorded as 6.61, and after three days of fermentation, it increased to 6.73-7.02 for xylose and 6.77-7.05 for glucose. A study has reported that this pH increase during photo fermentation may be due to the accumulation of the biopolymer polyhydroxybutyrate (PHB) formed by purple non-sulfur bacteria (PNSB) as one of the by-products [21]. In addition, for light fermentation by *Cereibacter sp.*, the addition of xylose appears to be optimal for accumulating higher biological hydrogen. The hydrogen formation was 19% higher for xylose than for glucose. A similar result was obtained in a photo fermentation experimental study, which reported that 4.73 mol H_2 /mol glucose and 5.24 mol H_2/mol xylose were obtained when glucose and xylose were utilized as the carbon source in light fermentation by *Rhodobacter sphaeroides* [22]. The higher pH resulting from glucose as the substrate suggests that PNSB may prefer glucose over xylose as the organic feedstock for biopolymer formation. Additionally, PNSB may prefer xylose for optimal biological hydrogen formation pathway.

Optimal conditions for dark and light hydrogen fermentation were achieved with 17.5 g/L and 10 g/L of xylose, respectively. The effects of metal ions on dark and photo fermentation will be investigated using the optimal carbon source. Transition metals have distinctive inorganic and redox properties that make them suitable as cofactors for enzymes. The unfilled d-orbitals of transition metals have made them redox-active, allowing metal cofactors to serve as structural and catalytic roles in biological metabolism [23]. The transition metals experimented with were 50μM of iron (II) sulfate heptahydrate, copper (II) chloride dihydrate, manganese (II) chloride tetrahydrate, zinc chloride, cobalt (II) chloride hexahydrate, nickel (II) chloride hexahydrate, and calcium chloride dihydrate. A controlled fermentation medium without the addition of metal ions will be utilized for comparison.

Biological hydrogen production from (A) dark fermentation and (B) photo fermentation based on various metal ions

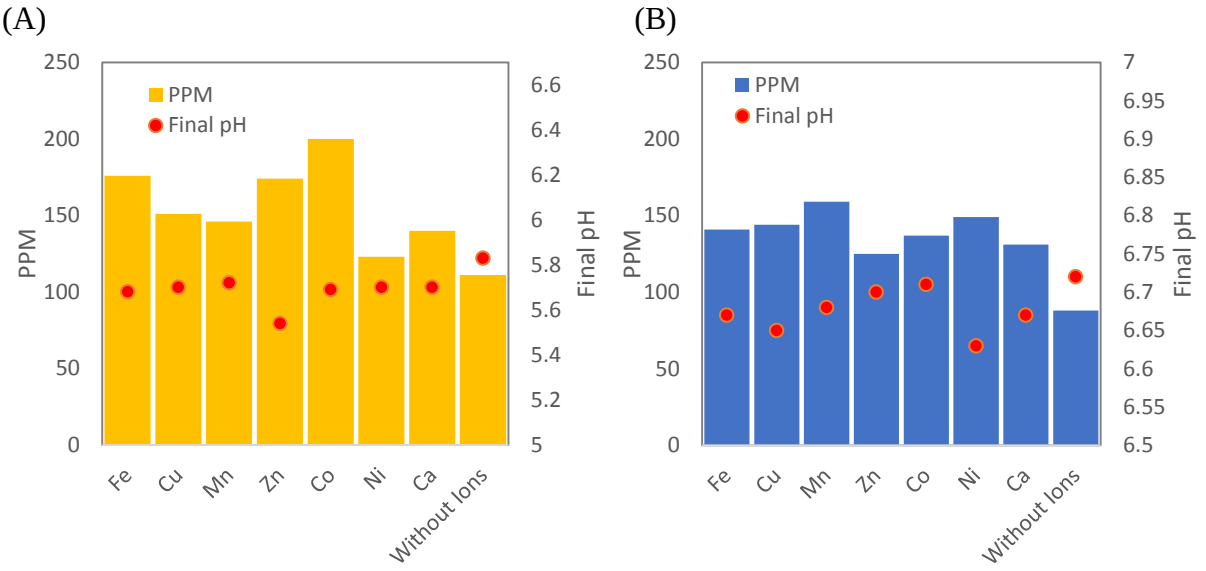

Figure 3.5: Cumulative hydrogen production by (A) *Bacillus sp.* and (B) *Cereibacter sp.* based on various micronutrients.

Figure 3.5 demonstrates the impact of various metal ions on the production of biological hydrogen through dark and photo fermentation. The results show that cobalt (II) chloride hexahydrate is the optimal metal ion for biological hydrogen production by *Bacillus sp.*, leading to a total cumulative biological hydrogen yield of 200ppm. During dark fermentation, the acids by-product produced may corrode cobalt (II) chloride hexahydrate, releasing Co^{2+} into the fermentation environment. These organic compounds in the process may reduce the metal ions, leading to an improved biochemical reaction with electron transfer that enhances the biological hydrogen evolution [24]. In comparison to the controlled condition without the introduction of any metal ions, the cumulative biological hydrogen increased by roughly 80% to 111ppm. For photo fermentation by *Cereibacter sp.*, the optimal micronutrient for biological hydrogen evolution was manganese (II) chloride tetrahydrate, which resulted in an accumulation of 159ppm of biological hydrogen after three days of fermentation. The hydrogen yield was enhanced by roughly 81% compared to the controlled fermentation medium. The optimal concentration of metal ions added can stimulate nitrogenase activity in photo fermentation, thereby increasing hydrogen formation. A similar result was obtained with the introduction of a 50μM concentration of Mn^{2+}, which enhanced the biological hydrogen yield by PNSB [25].

Furthermore, Figure 3.5 shows that all the fermentation media with micronutrients increased the accumulation of biological hydrogen compared to the controlled condition. The introduction of metal ions significantly enhanced the metabolism of bacteria during fermentation. For example, the controlled fermentation medium had a higher final pH than the other fermentation media with metal ions. Previous research has revealed that the addition

of micronutrients enhances the activity of PNSB, resulting in higher acid by-product production [26]. The activated carbon added can enhance the light fermentation process, leading to the formation of VFAs and ethanol by-products [27]. In addition, in dark fermentation, Co, Fe, and Ni metal ions can bind into the active site of hydrogenase, promoting electron transfer to hydrogenase and ferredoxin. Therefore, ferredoxin can act as an important redox mediator to convert pyruvate into VFAs and CO_2 due to its low reduction potential [24], [28], [29]. Consequently, including metal ions in the fermentation process results in a more acidic fermentation medium.

After studying the effect of substrate concentration and metal ions on the fermentation process, the inoculum size of bacteria was investigated for biological hydrogen formation. The xylose concentration used in the experiments was 17.5 g/L for dark fermentation and 10 g/L for light fermentation.

Biological hydrogen production from (A) dark fermentation and (B) photo fermentation based on various inoculum concentration

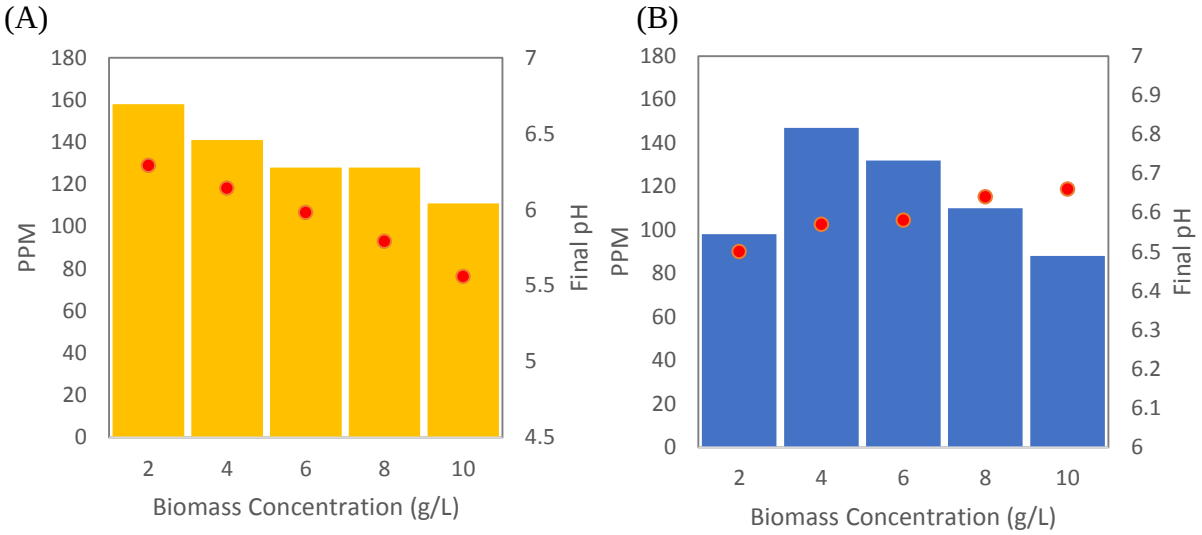

Figure 3.6: Cumulative hydrogen production by dark and photo fermentation based on various size of inoculum.

Inoculum size is a critical parameter in liquid-culture fermentation research, as it significantly affects various culture parameters such as cell growth rate, substrate utilization, and culture morphology [30]. Figure 3.6 demonstrates the influence of different inoculum sizes on cumulative biological hydrogen yield during dark fermentation. Results indicate that an inoculum size of 2 g/L is optimal for biological hydrogen formation, generating 158 ppm. During fermentation, the initial pH was recorded as 6.57, 6.55, 6.53, 6.51, and 6.46 for 2, 4, 6, 8, and 10 g/L inoculum size, respectively. As shown in Figure 3.6, higher inoculum sizes led to more acidic fermentation media after three days, with a final pH of 6.29, 6.14, 5.98, 5.78, and 5.56 achieved for inoculum sizes of 2 to 10 g/L. Wardani, Cahyanto, Rahayu, and Utami (2017) have reported that larger inoculum sizes result in faster acid production [31]. The low acid production observed for smaller inoculum sizes may be due to the low density of the starter culture. However, a higher acid concentration can increase the toxicity of the

fermentation media, leading to lower biological hydrogen yield. Therefore, a higher cell concentration results in higher VFA accumulation and lower biological hydrogen yield
In light fermentation, the optimum biological hydrogen yield accumulated was 147 ppm from a 4 g/L inoculum size of *Cereibacter sp.*. It is observed that the higher the inoculum concentration for light fermentation, the lower the biological hydrogen yield. PNSB captures light to perform complex biochemical reactions that synthesize intracellular molecules to sustain cell growth. Similar to biological hydrogen production, PNSB requires light to regulate the hydrogen metabolism pathway [32]. From the photo fermentation study, it is observed that the higher the inoculum size of PNSB, the higher the turbidity of the fermentation media after three days of fermentation. Therefore, the penetration of light into the fermentation media may be inhibited due to the high turbidity of the higher dry cell weight, which may affect the metabolism of PNSB in biological hydrogen formation. Shui et al. (2022) investigated the effect of light perturbation on biological hydrogen evolution from PNSB, using light perturbation parameters of 4000 Lux, 5000 Lux, and 6000 Lux. The study revealed that the maximum hydrogen yield achieved was 124.3 mL H_2/g TS from 6000 Lux light perturbation, compared to 104 mL H_2/g TS without light perturbation, resulting in a 20% enhancement in biological hydrogen yield [33]. This may be due to light perturbation making the light distributed uniformly in the fermentation reactor and increasing the area of PNSB exposed to light, thereby improving the rate of hydrogen production [34]. Thus, a higher initial concentration of PNSB resulted in a higher turbidity of fermentation broth, which limited the penetration of light into the photo fermentative media and reduced the rate of biological hydrogen production.

4. Conclusion

The DNA identification study confirmed the presence of *Bacillus paramycoides* (MCCC 1A04098) and *Cereibacter azotoformans* (JCM 9340) cells in the samples. The research revealed that process parameters such as type and substrate concentration, pH, type of metal ions, and inoculum size have significant impact on hydrogen production efficiency and stability. To advance this research work, exploration on metabolic pathway modification, genetic engineering enhancement, and co-culture hydrogen production capabilities can be proposed. Moving forward, the findings from both dark and photo fermentation experiments will be utilized as preliminary data for future co-culture investigations. Overall, the findings of this study have implications for the development of biodegradation technology, with potential for mitigating environmental issues associated with traditional hydrogen production methods. The discovery of a novel microbial strains with unique metabolic pathways also opens opportunities for further exploration of the diversity of microorganisms and metabolic pathways involved in biodegradation for hydrogen production.

5. Declaration of Competing Interest

The authors declare no conflict of interest.

6. Acknowledgment

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