Co-immobilization of Nano-Metal and *C. pasteurianum* for Dark Fermentation Anaerobic Hydrogen Production

Anongnart Wannapokin, Yu-Tzu Cheng, Sheng-Zhe Wu, Ping-Heng Hsieh, and Chun-Hsiung Hung

***Abstract*—In the suspension culture, the sharp structure of some nano-metals may destroy the cells and cause metal washout from the system, causing secondary pollution. A novel method of combination of nano-metals addition and immobilized dark fermentation microorganism can overcome this problem. In this study, the co-immobilization approach, nano-metal and the dark-fermented bacteria were incubated together within PVA-boric acid gel granule, could prevent washout of hydrogen producing microorganism as well as the added nano-metals. The results showed when 400 mg/L nano-metal nickel (NP-Ni) and 400 mg/L nano-metal iron (NP-Fe) were added within the immobilized cell, both the maximum H_2 production yield (HPY) and H_2 production rate (HPR) were improved to be 1.28 mol H_2/mol glucose, 3.13 H_2 L/L/day and 1.19 mol H_2/mol glucose, 1.90 H_2 L/L/day, respectively. The method is expected not only to allow microorganisms to have higher environmental tolerance but also increase hydrogen production.**

***Index Terms*—Hydrogen, dark fermentation, immobilization, nano-metal.**

I. Introduction

Nowadays, energy is indispensable in human life. The main source of energy is still from non-renewable energy sources, which has brought about environmental problems such as the greenhouse effect and abnormal climate. If we do not change the current habits and perceptions of using energy, the damage of the environment will become a catastrophic result, and it will result in the inability to recover. Therefore, the selection of energy has become a very important issue at present. Among the many alternative green renewable energy sources, the one energy source that receives special attention for responding to these requirements is hydrogen. Hydrogen has the most prospects and potential. What is the value of hydrogen with such high potential, because hydrogen only produces water after it is completely burned, it has clean and non-polluting properties to the environment, and it can provide a higher heat of combustion.

Dark fermentation anaerobic hydrogen production can adapt to whole weather conditions and not require light sources. It can use a variety of carbon sources to produce valuable metabolic by-products such as acetic acid, butyric acid and alcohols [1]. However, due to the low efficiency of dark fermentation bio-hydrogen production, several reports have tried to find ways to improve hydrogen production efficiency, so that hydrogen production systems can achieve optimal conditions [2].

In most of the bio-hydrogen production studies which are often used in the form of suspension culture. However, microorganisms are often washed out in continuous flow systems, resulting in unstable operating conditions and reduced hydrogen production [3]. Therefore, many studies use immobilization techniques to immobilize microbial cells to prevent loss of biomass, resulting in reduced microbial cell density and hydrogen production [4]. Immobilized microbial cells have the following advantages: bio-hydrogen production yield can be increased, it can survive under difficult conditions, increase substrate conversion rate, increased the operational ability at higher organic loading rates and shorter residence times, separation and filtration can be simplified, reduced processing costs, make microorganisms reusable, minimize contamination from microorganisms and protect microorganisms from shear stresses caused by agitation [4]. Hydrogen production is associated with cell growth, and high cell density should be a prerequisite for high hydrogen production. Immobilized cells can increase cell density in bioreactors resulting in increased hydrogen production [5].

Nanotechnology has excellent performance in many applications such as catalysis, optics, and medicine. In recent years. Application of nano-metal materials to a biological system is becoming popularization as it has unique physical and chemical properties. Currently, in order to overcome the limitation of low-hydrogen production by dark fermentation, nanoparticle (NP) like nano-metal or nano-metal oxides had been demonstrated to be able to enhance intracellular electron transfer and promote [NiFe]-hydrogenase and [FeFe]-hydrogenase activity and increase hydrogen production [6], [7]. In addition, many researchers have shown that the smaller nanoparticle size is better than the bigger one about the hydrogen production rate [8].

At present, most of the studies on adding nano-metal to dark fermentation bio-hydrogen production are based on suspension culture. In contrast, during suspension culture, the sharp structure of some nano-metals may destroy the cells and may cause the metal to wash out from the system, causing secondary pollution [9]. Therefore, this study attempts to co-immobilized nano-metals and *C. pasteurianum* for dark fermentation anaerobic hydrogen production, and observe the hydrogen production state of the anaerobic fermentation microorganisms.

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II. Material and Method

A. Microorganism and Culture Medium

The hydrogen production bacteria (HPB), *Clostridium pasteurianum* CH5, used in this study was isolated from a sugar-containing wastewater treatment system. Each month, 10 mL of pre-incubated bacterial liquid was transferred to a serum bottle containing 90 mL of fresh PYG matrix (German Species & Bacterial Preservation Center, Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ), and sealed the 125 mL serum bottle after replaced the headspace with argon gas to maintain the anaerobic environment. The PYG medium component contains 0.04 mg/L of K_2HPO_4 + KH_2PO_4, 0.0008 g/L of $CaCl_2$, 0.08 g/L of NaCl, 0.0192 g/L of $MgSO_4$•$7H_2O$, 0.04 g/L of $NaHCO_3$, 5 g/L of peptone, 5 g /L of tryptone, 10 g/L of yeast extract, 1.1 mg/L of $FeSO_4$•$7H_2O$ and 5 g/L of glucose, and the initial pH of the medium was adjusted to 7 [10].

B. Preparation of Fe (NP-Fe) and Ni (NP-Ni) Nanoparticles

Magnetic hematite nanoparticles, with a nominal size of 50-100 nm and 97% trace metal basis, were provided by SIGMA-Aldrich Corp. (St. Louis, MO, U.S.A.). Magnetic nickel with a nominal size of 30-50 nm and 97% trace metal basis, were provided by UniRegion Bio-Tech Corp. (Taiwan, R.O.C.).

C. Immobilization Method

The immobilization method selected for the experiment was the entrapment method which was carried out by referring to Wang et al. [11]. First, 3.5 g/L of Alginate and 0.5 g/L of κ-carrageenan were mixed with 30 mL of deionized water in the serum and sterilized in an autoclave (121 ℃). After sterilization, adding 7.0 g/L of polyvinyl alcohol with 10 ml of sterile water in the serum at laminar flow, and placed in an oven at 80 ℃ two hours, after the heating, shaking it appropriate time to evenly mix, then add CH5 bacteria solution (OD = 1, 50 mL concentrated to 10 mL) and nano-metal (Fe, Ni) at the laminar flow. Finally, extract 10 mL of all mixed colloids with a syringe, and add $CaCl_2$ of 1.8% (w/v%) and H_3BO_3 of 2% (w/v%) of the cross-linking agent, and placed in a refrigerator at 4 ℃ for 18-24 hours, to form diameter 3-4 mm of boric acid gel particles, and washed with sterile water several times, then stored in a refrigerator at 4 ℃ overnight.

D. Analysis of Hydrogen Production

The volume of the headspace gas of the batch bottle after the end of the final experiment was extracted with a gas volume of 0.5 mL gas volume, and the gas chromatography-thermal conductivity detector (GC-TCD) was used to analyze the composition of the inorganic gas generated in the batch bottle. In the experiment, the argon gas (Ar) is filled in the headspace of the serum. Therefore, the carrier gas in the GC-TCD cannot use argon gas but helium gas (He). The gas chromatograph used in the study was Agilent GC-7890A, the detector was a thermal conductive detector, the tube was 60/80 Carboxen-1000, the initial temperature was 35 ℃, and the temperature was raised to 20 ℃/min until 225 ℃, the carrier gas is He, the flow rate is 30 mL/min, the temperature of the injector is 100 ℃. The calibration curve is obtained by using a standard mixture gas (40% of H_2, 30% of CO_2, and 30% of CH_4) in different volumes (0.4, 0.5, 0.6, 0.8 and 1.0 mL) after analysis by gas chromatography. The signal area and gas volume are plotted as a calibration curve.

III. Results and Discussion

A. Comparison of Immobilized and Suspended Microorganism on Hydrogen Production by Dark Fermentation

After 96 hours of incubation, the cumulative biogas production and hydrogen production of immobilized CH5 (control), the CH5 with nano-metals co-immobilized, and the suspended CH5 are shown in Fig. 1 and Fig. 2.

Results showed that the cumulative gas production of CH5 after gel immobilization was better than the suspended ones, regardless of whether the nano-metals was added or not. As shown in Fig. 2, the hydrogen production of immobilized CH5 is also significantly better as well, which can increase hydrogen production to about 16%. The amount of carbon dioxide production was less than that in suspension form. In another research, when the retention time is controlled at 24 hours, the hydrogen production rate of the immobilized anaerobic sludge and suspension state is 2.10 and 0.99 mol H_2/mol respectively. This is in agreement with what had been reported that the immobilized approach performed better [12]. The research of Wu et al. indicated that using sucrose to be carbon source, the result of hydrogen production rate about immobilized other strain of *Clostridium* sp. and suspension state is 21.3 and 6.8 mmol/L/h respectively, that is three times more than suspension state under batch experiment of immobilization and suspension state by fermentation [13]. These research are consistent with the results of our research, it can be proved that the immobilized *Clostridium* sp. can indeed increase hydrogen production.

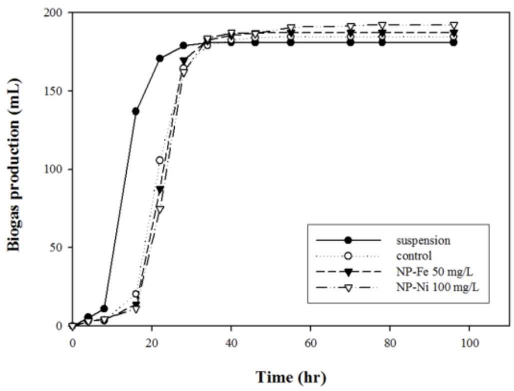

Fig. 1. Accumulative biogas production of immobilized CH5 and suspended CH5.

However, in the case of cell immobilization, there is no significant difference in accumulative biogas production and hydrogen production with or without nano-metal addition. Therefore, it is questionable that co-immobilization of CH5 and nano-metal did improve hydrogen production as previously proposed. Hsieh et al. indicated that nano-metal improved electron transfer after directly contacting the microbial cell membrane, thereby increasing the electron transfer rate between hydrogenase and ferredoxin, and improving hydrogen production [9]. Therefore, the subsequent processing conditions for the preparation of the granules will be adjusted in the next step to find a suitable preparation method and establish an optimum granule preparation operating condition.

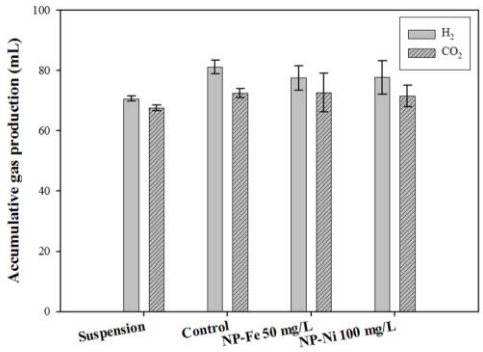

Fig. 2. Accumulative gas composition of immobilized CH5 and suspended CH5.

B. Establishing Optimal Granule Preparation Conditions

The purpose of this study is to establish a better granule preparation procedure for hydrogen production. Difference between gel mixed with nano-metal before adding microorganisms (CH5 is not directly contacted with the nano-metal) and pre-incubating nano-metal with CH5 before adding gel (CH5 contacted with the nano-metal directly) was examined to study the effect of stimulation. NP-Ni was selected as the testing metal at different concentrations of 0 (control), 100, 200, 300 and 400 mg/L.

1) Gel mixed with nano-metal before adding microorganisms

After 96 hours of culture, there is little difference in the group of NP-Ni and control, but significantly different was observed when adding 200 mg/L NP-Ni. Its cumulative biogas production improved up to 28% more than the control group (Fig. 3). But it can not improve hydrogen production (Fig. 4). Moreover, the standard deviation in the batch of three repeat groups is rather large and unstable. Therefore, it is speculated that the granules prepared by this method cannot effectively contact with CH5 and nano-metal.

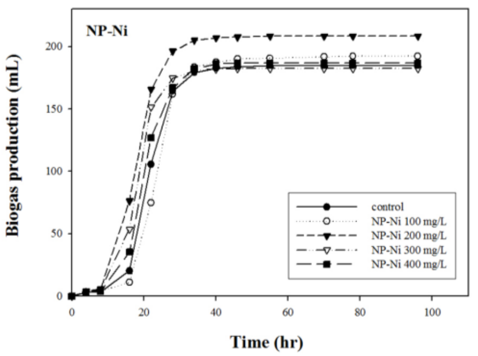

Fig. 3. Accumulative biogas production of gel mixed with nano-metal before adding CH5.

2) Pre-incubating CH5 with Nano-metal before adding gel

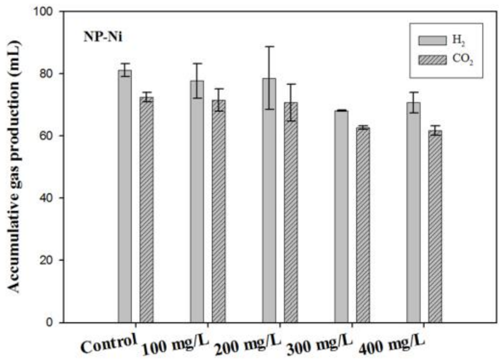

Fig. 4. Accumulative gas composition of gel mixed with nano-metal before adding CH5.

After 145 hours of incubation, it was found that accumulative biogas production of adding NP-Ni is better than the control group for every tested concentration. Adding 100-400 mg/L NP-Ni can improve 13-32% of accumulative biogas production more than the control group (Fig. 5). On the cumulative hydrogen production, 36-51% improving were observed than the control group (Fig. 6). Moreover, the standard deviation in the batch of three repeat groups is small and stable. It is speculated that pre-incubated CH5 with nano-metal before making gel granules is the right approach for this proposal as illustrated in Fig. 7.

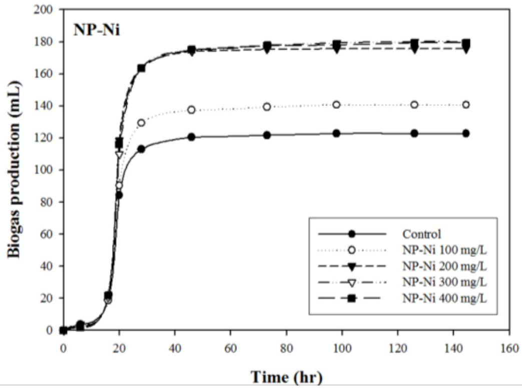

Fig. 5. Accumulative biogas production of nano-metal mixed with CH5 before adding gel.

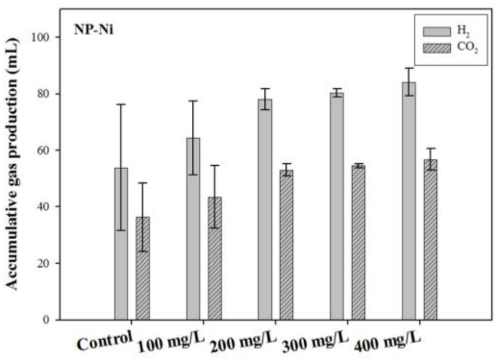

Fig. 6. Accumulative gas composition of nano-metal mixed with CH5 before adding gel.

C. Performance of Metal-Microorganism Pre-incubated Method under Different Concentrations of Nano-Metal

1) NP-Ni as added metal

After establishing the metal-microorganism pre-incubated method for granular preparation, all the following experiment were performed with this approach. The cumulative gas production was improved to 13%, 30%, 32%, and 32% respectively when adding 100, 200, 300 and 400 mg/L NP-Ni, the maximum cumulative biogas production is 180.0 mL when adding 300 mg/L NP-Ni. And the cumulative hydrogen production was improved 36%, 47%, 49%, and 51% respectively, and the maximum cumulative hydrogen production is 84.1 mL when adding 400 mg/L NP-Ni. It can be seen from the above that as long as the concentration of nano-metal Ni higher than 200 mg/L, hydrogen production will be improved as shown in Fig. 8 and Fig. 9. However, according to the study of Hsieh, when adding a higher concentration of NP-Ni into suspended dark fermentation hydrogen production system, will lead to a decrease in cumulative biogas production and hydrogen production [9]. In this study, co-immobilized with NP-Ni and CH5 in the granule did not cause negative hydrogen production under high concentration of NP-Ni addition. It was speculated that this proposed granular preparation might reduce the toxicity of high concentration of nano-metal addition.

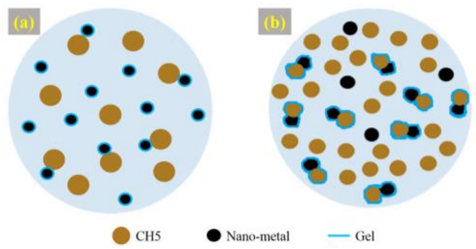

Fig. 7. Comparison of differences in operating conditions of granules preparation (a) gel mixed with nano-metal before adding CH5 and (b) nano-metal mixed with CH5 before adding gel.

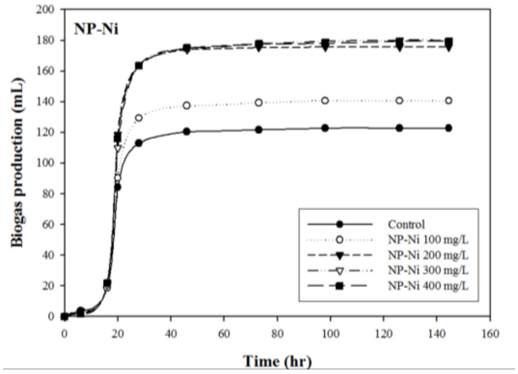

Fig. 8. Accumulative biogas production of different concentration of NP-Ni.

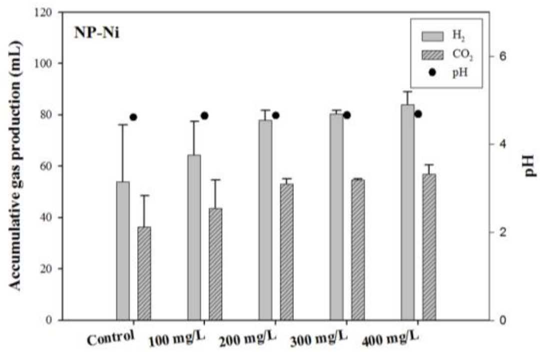

Fig. 9. Accumulative gas composition of different concentration of NP-Ni.

When the addition of NP-Ni concentration is 0 (control), 100, 200, 300 and 400 mg/L, the value of hydrogen production yield was 0.62, 0.97, 1.18, 1.22 and 1.28 mol H_2/mol glucose respectively. It showed that co-immobilized with CH5 and NP-Ni can also improve hydrogen production yield, and the maximum value is 1.28 mol H_2/mol glucose when adding 400 mg/L NP-Ni. The hydrogen production rate from low to high concentration of NP-Ni addition is 2.20, 2.87, 2.98 and 3.13 H_2 L/L/day respectively. It increased when the concentration of NP-Ni was higher. All of these production rates were better than the control group (1.88 H_2 L/L/day). In terms of gas composition, it does not change under different concentration of NP-Ni addition, and the average percentage of hydrogen gas is greater than 44%.

2) NP-Fe as added metal

The cumulative biogas production was improved up to 15%, 9%, 15%, and 16% respectively when 50, 100, 200 and 400 mg/L NP-Fe were added. Maximum cumulative biogas production was 179.0 mL when adding 400 mg/L NP-Fe. Cumulative hydrogen production was improved up to 22%, 18%, 27%, and 27% respectively, and the maximum cumulative hydrogen production was 74.2 mL when adding 400 mg/L NP-Fe. It can be seen from the results that as long as 50 mg/L NP-Fe or more were added, improved hydrogen production was observed as showing in Fig. 10 and Fig. 11.

However, according to the study of Hsieh *et al.*, when a higher concentration of NP-Fe was added into a suspended growth of dark fermentation hydrogen production system, accumulative biogas production and hydrogen production decreased significantly [9]. This inhibition was not observed in this study under high concentration of NP-Fe addition. This is similar to the previous result on NP-Ni as this proposed granular preparation indeed could reduce the toxicity of higher concentration of nano-metal addition.

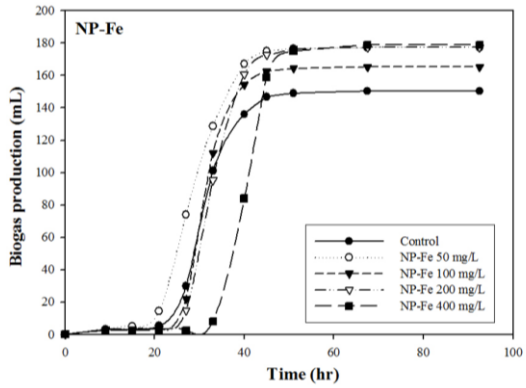

Fig. 10. Accumulative biogas production of different concentration of NP-Fe.

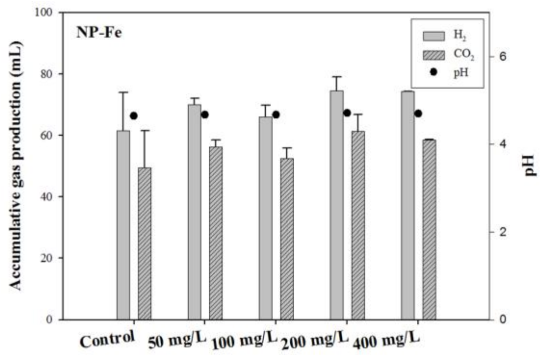

Fig. 11. Accumulative gas composition of different concentration of NP-Fe.

When the addition of NP-Fe concentration is 0 (control), 50, 100, 200 and 400 mg/L, the value of hydrogen production yield was 0.86, 1.09, 1.05, 1.19 and 1.19 mol H_2/mol glucose respectively. It showed that added NP-Fe can improve hydrogen production yield, and the maximum value is 1.19 mol H_2/mol glucose when adding 200 and 400 mg/L NP-Fe. Hydrogen production rate from low to high concentration NP-Fe addition is 1.53, 1.46, 1.70 and 1.90 H_2 L/L/day respectively. All of these production rates were better than the control (1.29 H_2 L/L/day). Ratios of gas composition in a different concentration of NP-Fe addition were about the same and the average of hydrogen gas percentage is about 39%.

IV. Conclusion

The purpose of this study is to test the feasibility of co-immobilization of nano-metal and *C. pasteurianum* CH5 for dark fermentation anaerobic hydrogen production. Different methods of granules preparation were tested. Accumulative biogas production and the cumulative hydrogen production in immobilized form were all better than in the suspended form, and the immobilized CH5 could increase a 16% of hydrogen production. The better-immobilized protocol could be achieved when nano-metal and microorganisms were incubated together before being made to gel granules. The maximum cumulative biogas production of nano-metal nickel (NP-Ni) was 32% higher than the control group, and the cumulative hydrogen production was 51% higher than the control. The maximum cumulative gas production yield of nano-metal iron (NP-Fe) increased by 16% compared to the control group, and the cumulative hydrogen production increased by 27% compared with the control group. Overall, this co-immobilization approach, nano-metal and the dark-fermented bacteria were incubated together within PVA-boric acid gel granule, could benefit the hydrogen production and prevent washout of hydrogen producing microorganisms, which shows great potential in application of co-immobilization of CH5 and nano-metal.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Anongnart Wannapokin, Yu-Tzu Cheng and Sheng-Zhe Wu concuted the research, analyzed the data and wrote the paper. Ping-Heng Hsieh, and Chun-Hsiung Hung guidelines and instructive.

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