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Research Article

Biohydrogen Production by Reusing Immobilized Mixed Culture in Batch System

Astrilia Damayanti^a, Sarto^b, and Wahyudi Budi Sediawan^b

^aDepartment of Chemical Engineering, Universitas Negeri Semarang, Kampus Sekaran, Gunungpati, Semarang, Indonesia

^bDepartment of Chemical Engineering Universitas Gadjah Mada, Yogyakarta, Indonesia

ABSTRACT. Biohydrogen production via dark fermentation is a prospective renewable energy technology. In the process, reused of immobilized mixed culture is very important as their activities greatly influence hydrogen production. The aim of this work was to evaluate the reuse of alginate beads affecting the biohydrogen production for 45 days. This study in batch reactor were performed using glucose 10 M as substrate, alginate and activated carbon as immobilization matrix materials, chicken eggshell as buffer, and cow dung bioreactor as mixed culture. Hydrogen and pH on fermentation product are investigated by gas chromatography (GC) technique and pH meter, respectively. The colony diameter on immobilized and co-immobilized mixed culture was observed using optical microscope and colony diameter was measured using Image-Pro Plus Software v4.5.0.29. The surface morphology of immobilization and co-immobilization beads were determined using scanning electron microscope (SEM). The results showed that the colonies growth observed using optical microscopy or SEM was apparent only in the immobilization of mixed culture. The average growth and diameter of colonies per day were 90 colonies and 0.025 mm, respectively. The weight of beads and pH during the 45-day fermentation process for bead immobilization of mixed culture were 1.32–1.95 g and 6.25–6.62, correspondingly, meanwhile, the co-immobilizations of the mixed culture were 1.735–2.21g and 6.25–6.61, respectively. In addition, the average hydrogen volume of glucose fermented using an eggshell buffer and reusing the immobilization and co-immobilization beads was 18.91 mL for 15 cycles. ©2020. CBIOR-IJRED. All rights reserved

Keywords: biohydrogen; reused beads; immobilization; mixed culture; batch

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1. Introduction

The identification of an alternative energy is challenging given the depletion of fossil fuels amount. This depletion was due to energy demand which has increased along with the human population and the combustion effects of greenhouse gases that have caused global climate change. One of potential renewable and non-polluting alternative energies to be developed is hydrogen (Penniston and Kana 2018; Akil and Jayanthi 2015; Reyhani and Zilouei 2013). In addition, hydrogen has the highest heating value (143 GJ/ton) among the sources of energy (Das 2009).

The most common biohydrogen is produced through dark fermentation technology because its temperatures and pressure can be conducted moderately (Akil and Jayanthi 2015). Moreover, the production of biohydrogen uses minimal energy input (Sekoai et al. 2017). The use of naturally mixed culture for hydrogen fermentation production offers numerous advantages over single culture considering the metabolic flexibility and robustness of the former process (Wang et al. 2017). An acid pre-treatment method was used to prepare considerable anaerobic hydrogen-producing microbial

from natural sources (Cai and Wang 2016; Cheong *et al.* 2006). In addition, this method also reported to help suppress the methanogenic bacteria activity (Saripan and Reungsang 2014). Furthermore, an enriched (natural) mixed culture was used to enhance the yield and production rate of hydrogen (Sivagurunathan *et al.* 2014).

The use of immobilized cell systems in developing a biohydrogen process development has several advantages over suspended cell systems. These advantages include improved handling (Penniston and Kana 2018), resistant to cell washout, production of enhanced yields, and reusability (Sekoai, Yoro, and Daramola 2016). Technology by entrapping cells in a solid matrix through dripping extrusion is a common approach to immobilising cells.

Alginate is a natural polysaccharide and is preferred as an immobilising microorganism considering its high transparency (Ng *et al.* 2017), nontoxicity (Hassan *et al.* 2014) and ability to live within small holes (Covarrubias *et al.* 2012). However, alginate beads have weak mechanical stability because they do not retain their structure at high cation concentrations (K^+ , Ca^{2+} and Mg^{2+}) (Sekoai et al. 2017). Co-immobilized activated

*Corresponding author: astrilia.damayanti@mail.unnes.ac.id

carbon (AC) in alginate is a technique that improves mechanical strength (Mesran *et al.* 2014). AC is an immobilization carrier and has been well-documented as a support matrix in dark fermentation (Zhang *et al.* 2017). AC has a high specific surface area and low toxicity. Its character sustains cell viability, where a fermentative inoculum can grow freely inside a porous structure on the surface (Zhang *et al.* 2017)(Jamali *et al.* 2016). Highly porous structure and the surface, where the bacteria can grow freely, sustain cell viability and enhance cell density (Zhang *et al.* 2017) (Jamali *et al.* 2016). The simultaneous use of two or more matrix materials is called co-immobilization (Siahpush *et al.* 1992).

Biohydrogen generation through dark fermentation is largely depends on pH level, as the pH affects enzyme activities and metabolite transport (Davila-Vazquez *et al.* 2011). Moreover, it determines product distribution during fermentation (Muñoz-Páez *et al.* 2014). A low pH can inhibit hydrogen-consuming methanogenic microorganisms (Wu, Yao, and Zhu 2010) given the generation of organic acids (Zhu *et al.* 2009). An alternative is to add sufficient buffer materials or bicarbonate. A natural bicarbonate can be obtained from chicken eggshell which contains 93.6% CaCO₃ (Neunzehn *et al.* 2015).

A material requirement for cell immobilization is suitable for cell regeneration (Dzionek *et al.* 2016). Hydrogen is mainly produced during cell growth (Luo *et al.* 2010). Therefore, the major objective of this study is to evaluate the gel strength of mixed culture immobilization and co-immobilization (Shoichet *et al.* 1996) on anaerobic biohydrogen using reused beads, a new medium and chicken eggshell. Furthermore, the size and amount of microbial growth in immobilization and co-immobilization were used to describe the hydrogen production. In addition, the pH and bead weight were evaluated during the fermentation.

2. Materials and Methods

2.1. Anaerobic mixed culture and pre-treatment

An anaerobic mixed culture obtained from cow dung biodigester (3.6 g VSS/l), Yogyakarta, Indonesia, was used as the inoculum. The inoculum (150 ml) was subjected to acid treatment by adjusting pH to 3 using 2M HCl (Merck, 37%) and maintained under anaerobic conditions for 24 h. Subsequently, the pH of acid pre-treated mixed culture was re-adjusted to 6 using 2 M NaOH.

2.2. Enrichment of hydrogen-producing mixed culture

Enrichment of hydrogen producing mixed cultures was conducted in 100 mL serum vials with 45 mL working volume. The pre-reduced anaerobically sterilized peptone-yeast extract-glucose (PYG) medium contained the following nutrients (in g/L): 10 glucose, 10 yeast extract, 10 peptones, 0.5 L-cysteine-HCl, and 0.001 resazurin. Mixed culture 2 ml was fed to a PYG medium bottle and then incubated for 24 hours. This method was repeated thrice. The final enriched mixed culture was used as an inoculum for the fermentation experiments.

2.3. Substrate and Medium composition

The medium composition consisted of 10 g/L peptone, 10 g/L yeast extract, 0,001 g/L resazurin, and 0,5 g/L L-cysteine-HCl. The substrate was 10 g/L glucose,

2.4. Immobilized and co-immobilized mixed culture

Immobilized material characteristics was similar to those in the previous experiment (Damayanti *et al.* 2018). Two grams of alginate was dissolved in 100 mL distilled water. The ratio of Alginate concentration to AC was 1:1. The preparation of immobilized and co-immobilized mixed culture was similar to that in the previous experiment (Damayanti *et al.* 2018)

2.5. Buffer from Chicken Eggshell

Powder of eggshells approximately 3.2 g dry weight was added into the fermentor. The chicken eggshell was prepared in accordance with the previous experiment (Damayanti *et al.*, 2017).

2.6. Biohydrogen Production Process

The dependent variables studied include total gas produced, amount of hydrogen, weight beads, and size of colony diameter. Two sets of fermentation processes were conducted in a 100-mL serum vial with 50 mL working volume under a sterile and anaerobic condition at 37 °C. The reactors were fed with 30% (v/v) substrate, 60% (v/v) medium and 10% (v/v) mixed culture. The bead weight and hydrogen-producing consortia of immobilization were three g and 0.3945 g. L⁻¹, respectively, whereas 3.5 g and 0.635 g. L⁻¹ for co-immobilization. Chicken eggshell, substrate, and medium were sterilised by autoclaving at 121 °C for 15 min.

The biohydrogen was processed through fermentation for three days per cycle or run. New glucose substrate and eggshell were used, but immobilization and co-immobilization materials were reused.

Hydrogen production was performed in batch system using glucose substrate, beads, and eggshell. The eggshell, immobilized and co-immobilized beads were required, similar to the previous experiment (Damayanti *et al.* 2017). The reactor and fermentation were conducted anaerobically (Damayanti *et al.* 2017). Each fermentation was rendered for three days. However, on the third day, the substrate was replaced, and the bead was restored. The process was performed for 45 days, and sampling was conducted in duplicate.

2.7. Visualization of colonization by optical microscopes and scanning electron microscope (SEM)

The measurement of colony diameter on immobilized and co-immobilized mixed culture was observed using an optical microscope (Celestron 44348 Pentaview Digital Microscope, China). Then, colony diameter was measured using Image-Pro Plus Software v4.5.0.29. A 0.112 mm cable was used as a standard. Furthermore, the actual colony diameter was calculated using MS Excel.

The surface morphology of immobilization and co-immobilization beads was observed using an SEM (JOEL, JEM-1200EX, Japan) at the magnification of 500 times. Colony observations on immobilization and co-

immobilization beads were initiated by fixation in a Bunsen flame and then coated with platinum. The samples were observed under an SEM (JOEL, JEM-1200EX, Japan).

2.8. Analytical methods

Hydrogen was analysed using gas chromatography (GC). Shimadzu GC 8A (Japan) equipped with a thermal conductivity detector (TCD) and molecular column sieve 5A (MS-5A) with 5m column length. Temperature of column, detector, and injector were set at 60°C, 70°C, and 70°C, sequentially. Nitrogen was used as carrier gas with an inlet pressure of 100 kPa. The pH of the sample during the fermentation process was measured using a Lutron PH-208 pH meter that was calibrated with buffer solution pH 7.

3. Results and Discussion

3.1. Colonies growth in immobilized and co-immobilized mixed culture

Observations of colony growth under immobilized and co-immobilized mixed culture conditions using optical microscopy were presented in Figures 1 and 2.

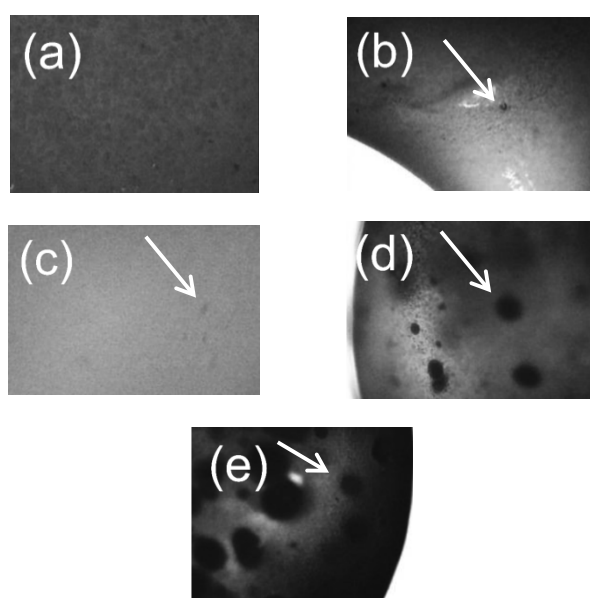


Fig.1. Growth of colonies on immobilized mixed culture observed using optical microscope (a) on zero-day at 100 times magnification, (b) on the 3rd day at 100x magnification, (c) on the 9th day at 100 times magnification, (d) on the 18th day, and (e) on the 21st day at 40 times magnification, respectively.

Figure 1 illustrates the growth of colonies on the surface beads of immobilized mixed culture. Starting with Figure 1a, taken on day 0, only a few or almost no colony could be seen as it was the beginning. Observation was continued on the third-day and ninth-day (Figures 1b and 1c) and images obtained shows that the colonies started to grow. Until the 18th and 21st day, the development of the number of colonies was increasingly seen as can be seen in Figures 1d and 1e. This shows that

the colony in this experiment can grow continuously even with a slow speed.



Fig.2. Co-immobilized mixed culture on 36th day, observed using optical microscope using 40 times magnification.

Figure 2 demonstrates that alginate was found in the outermost layer of the beads and in a black colour. This result was similar to that obtained by Mesran et al. (2014). The microorganism colonies in the form of a circle represent an aggregation of microorganisms as the result of alginate adsorption (Alonso et al. 2015). The diameter and number of colonies at a certain time are showed in Figure 3.

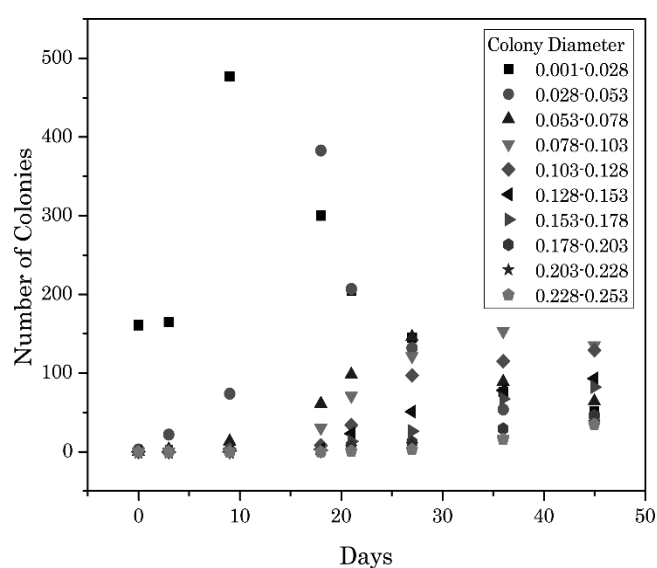


Fig.3. Profile of diameter and number of the colonies

Figure 3 presents that the indicators of colonisation growth include an extensive fermentation period and an increase in the number of colonies. The phenomenon of colony growth in immobilization and co-immobilization was due to the size of glucose (2,845 nm [ChemSketch software]) which was twice smaller than the pore size of alginate beads (5–200 nm) (Alonso et al. 2015). Thus, glucose was easily entered the pores of alginate beads and consumed by the bacteria. The morphology of beads with and without the colonies on day 0 and day 18th of fermentation for immobilized and co-immobilized beads was observed using the SEM (Figures 4 and 5).

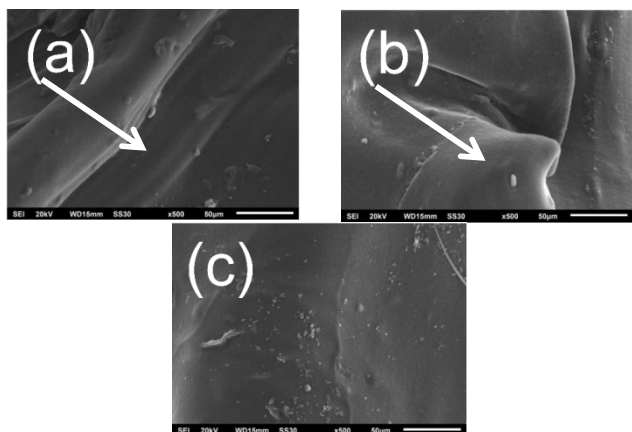


Fig.4. Morphology of SEM at 500 times magnification for immobilized beads (a) without colonies, (b) with colonies on day zero, (c) with colonies on 18th fermentation day

Figure 4 depicts that the growth of colonies is characterized by spherical protuberances on the surface of immobilized mixed culture from Day 0 (Figure 4b) to Day 18th (Figure 4c) in comparison with immobilized mixed culture without microorganisms (Figure 4a). The spherical protuberances are the colonies of microorganisms found in immobilized beads of many cavities that were used with the increase in microorganisms. These cavities were also the result of immobilization processes and serve as a physical separator between inhibitors and microorganisms (Covarrubias *et al.* 2012).

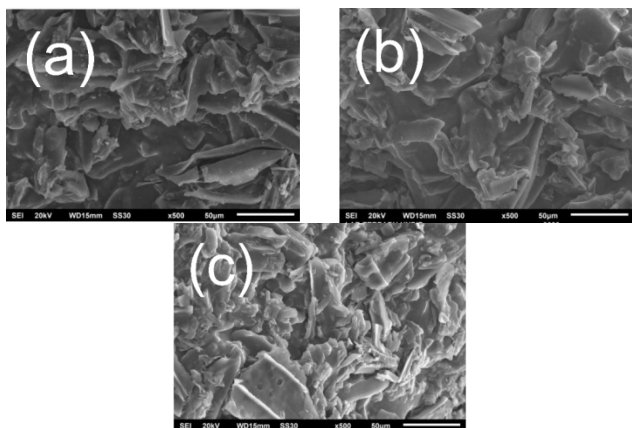


Fig. 5. Morphology of SEM at 500 times magnification for co-immobilized beads (a) without colonies, (b) with colonies on day zero, (c) with colonies on 39th fermentation day

Moreover, Figure 3 illustrates the surface of the immobilized mixed culture which is a rigid and solid layer that serves as the ultimate protection for microorganisms (Covarrubias *et al.* 2012). This type of surface was produced instantaneously whilst the alginate liquid drops into the cation-binding solution (e.g. CaCl₂ in this study) during polymerisation (Smidsrod and Skjåk-Braek 1990). This process was called an entrapment that causes more cavities beneath the surface layer than inside the beads (Zohar-Perez *et al.*

2004). Simultaneously, the shrinking surface layer processes of the beads caused the occurrence of spherical protuberances (Covarrubias *et al.* 2012).

Figure 5 demonstrates that the presence of microorganisms in colonies at the co-immobilization beads could not be acknowledged clearly. The structure of AC appears to be increasingly dominant. The co-immobilization in this study was similar to that obtained by Mesran *et al.* (2014), that is, the microorganisms were found in an AC hole.

3.2. Comparison of weight beads between immobilized and co-immobilized beads

Beads were reused to determine the weight of immobilization and co-immobilization beads during fermentation, as presented in Figure 6.

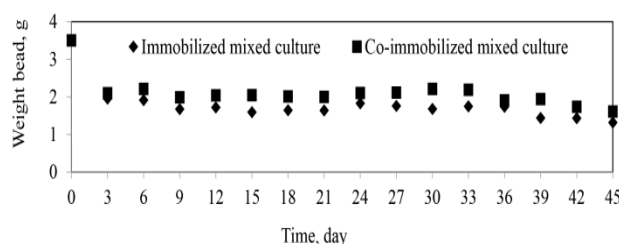


Fig. 6. Beads weight during the fermentation process

Figure 6 shows that the bead weights decreased on Day 3. However, the weight loss was greater in the immobilized mixed culture than in the co-immobilized mixed culture, thereby demonstrating that AC acts as an alginate buffer (Wu *et al.* 2002). The weight loss of immobilized and co-immobilized mixed culture was caused by the broken beads. Statistical analysis using single factor analysis reports p-value of 0.05167 which means p-value>0.05. This number means that the difference in material (immobilized and co-immobilized) does not make a significant difference in the weight of the bead obtained.

3.3. Comparison of pH between immobilized and co-immobilized beads

The level of acidity (pH) during fermentation using the reused beads for immobilized and co-immobilized beads was obtained, as exhibited in Figure 7.

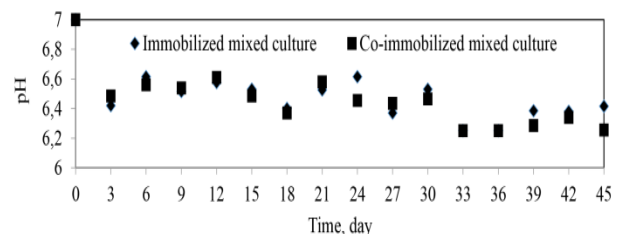


Fig. 7. pH solution during the fermentation process

Figure 7 shows that the pH declined from 7 to 6.4 on Day 3 and was subsequently constant at an average of pH 6.5 until Day 81. Thus, the chicken eggshell powder effectively retains the fermentation pH. The use of buffer

made of carbon dioxide/hydrogen carbonate/carbonate can prevent acidification (Deublein and Steinhauser 2008).

3.4. Reuse of beads intensity in biohydrogen production

The biohydrogen production of the reused-bead is displayed in Figure 8.

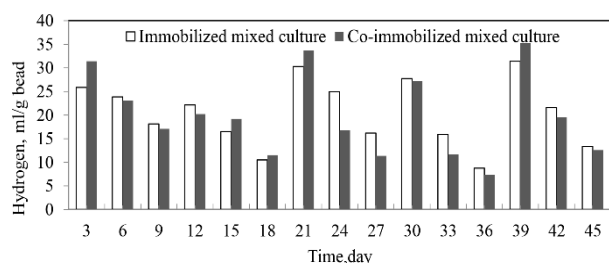


Fig.8. Biohydrogen production using immobilized beads and co-immobilized beads

Figure 8 shows that the biohydrogen gas produced are 1.05 times larger using co-immobilized beads than immobilized beads. Biohydrogen production in both beads visually fluctuated for 15 cycles. These fluctuating biohydrogen yields were similar to those obtained by Hu *et al.* (2007).

The biohydrogen production was evidently larger in immobilization beads than in co-immobilization beads on Days 6, 9, 12, 24, 27, 30, 33 and 36. The presence of an unfavourable bacterial community structure or mass transfer limitations was assumed to arise from co-immobilized cells. Therefore, bead leakage was further caused microbes to enter into a medium solution for these microbes to consume glucose by reusing immobilization beads, easier than co-immobilization beads (Mesran *et al.* 2014).

The production of biohydrogen produced by immobilized and co-immobilized mixed culture was relatively stable until 60 days (Figure 8). The stability of the biohydrogen production using immobilized beads was similar to the previous research, where the stable immobilization beads can produce biohydrogen until 60 days (Kumar *et al.* 1995). Therefore, the immobilized mixed culture could be used repeatedly. The pH sensitivity from acidic metabolite accumulation for 22 days of fermentation was due to the loss of calcium ions, thereby resulting in transparent, partially broken and soft immobilized mixed culture (Hu *et al.* 2007). The beads become brittle and finally broken down when eroded (Hu *et al.* 2007).

4. Conclusion

The colony growth during the anaerobic fermentation process by reusing immobilized mixed culture beads results in an increase in the number of remarkable colonies by 66% on Day 9. The mean weights of the immobilization mixed culture beads for 45 days were 1.78 and 6.45 g, whereas those of the co-immobilization mixed culture beads were 2.1 and 6.46 g. The biohydrogen productions in the immobilization and co-immobilization mixed culture beads were 19.20 and 18.61 mL/g bead.

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