Review Calcium Alginate Beads as Immobilizing Matrix of Functional Cells: Extrusion Dripping Method, Characteristics, and Application

Review Calcium Alginate Beads as Immobilizing Matrix of Functional Cells: Extrusion Dripping Method, Characteristics, and Application

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Abstract. Sodium alginate is the polymer matrix most commonly used for the immobilization of cells, enzymes, and microalgae for various purposes. One of the bead immobilization preparations is the droplet extrusion method in which $CaCl_2$ is the adsorbent. However, the use of $CaCl_2$, which is a cross-linking agent, can increase alginate susceptibility. Therefore, this review aims to provide an overview of the application of immobilized cells in the form of reused beads for the production of biohydrogen and bioethanol, as well as beads for removing heavy metals from wastewater, and removing potassium from vinasse. Meanwhile, the immobilized cells used were cow dung, Saccharomyces cerevisiae (*S. cerevisiae*), and *D. subspicatus*. All reported applications have shown that the initial bead shape of the drip extrusion method was spherical. However, over time the alginate beads become eroded due to repeated use. Round beads occurred when using 2% alginate concentration and the performance was optimum compared to 1% and 2% of alginate concentrations even though the cross-linked concentrations varied.

1. Introduction

The needs for both fuel as an energy source and the generation of industrial wastewater have steadily increased in line with the rapid growth of the world's population. However, industrial wastewater still offers great potential for use as microalgae culturing medium [1]. Recently, researchers have been attracted to search the method to produce fuels from organic wastes, such as rotten orange pulp waste for biohydrogen production[2], bioethanol synthesis from oil palm empty fruit bunches [3], and microbial biodegradation of hazardous pollutants. Unfortunately, the presence of limonene in orange pulp waste has been found to inhibit biohydrogen production[4]. Similarly, the high ethanol concentration could be the inhibitor for yeast in glucose fermentation to produce[5]. Such hazardous chemical concentration may also harm the growth of microalgae [1] and human life[6]. Considering those aforementioned phenomena, the immobilization of microbes on alginate matrices can be a promising option.

Alginate is commonly used as an alternative for immobilization, due to its desirable characteristics, such as superior chemical stability, non-toxicity, and low cost combined with incorporation efficacy[7]. However, alginate also suffers some serious drawbacks especially due to low stability, high porosity that exhibits rapid substrate and product diffusion, and low compatibility to some phenolic compounds, which may harm the living cells [8]. Gelation and gel mechanical properties of alginate are strongly dependent on the composition and concentration [9-10]. Sodium alginate concentration 2% w/v is used for alginate bead forming because it's a ball shape, adequate texture, and homogenity[9].

One of the recommended methods to prepare alginate beads is by extrusion dripping, which mainly due to its low cost, simplicity of its operation, and high cell viability[10], and further stabilized with an aqueous solution of calcium chloride [11]. This review elaborates the preparation and characterization of alginate beads for microbe immobilization using extrusion dripping including alginate beads utilization for production of biohydrogen by reusing of immobilized mixed culture for fermentation of glucose from oil palm empty fruit bunch in bioethanol synthesis, removal of heavy metals from industrial wastewater, and removal of potassium in vinasse.

2. Materials And Method

2.1 Production of biohydrogen by reusing immobilized mixed culture and bead analysis[12]

Forty-five milliliters of enriched-mixed culture obtained from biodigestion of cow dung (3.6 g VSS/l), Yogyakarta, Indonesia, was centrifuged at 4000 rpm for 10 minutes. The cell residue was resuspended twice in 10 mL of 0.97 % w/v NaCl. The mixed culture was mixed gently with 2 g sodium alginate (2% w/v) and 100 mL of 0.97% w/v NaCl to obtain a homogeneous mixture. Then, the mixed culture-alginate were sucked by a graduated syringe and dispersed dropwise into 0.1 M $CaCl_2$ solution to form beads. The beads in the $CaCl_2$ solution were stored at 4°C and washed with distilled water before further uses. The beads were subsequently used for hydrogen production in 102 days with glucose as a substrate and chicken eggshell as a buffer are replaced every 3 days. Colonies in immobilized mixed culture were analyzed by an optical microscope (Celestron 44348 Pentaview Digital Microscope, China) with 40 and 100 times magnification.

2.2 *Saccharomyces cerevisiae* (yeast) immobilization for ethanol production from synthetic glucose of Oil Palm Empty Fruit Bunches hydrolysis and bead analysis

Synthetic glucose concentration was 172 g / L [13]. *S.cerevisiae* (yeast) immobilization was performed by operating the syringe pump with a fluid of rate at 0.25 $mL.min^{-1}$. Ratio cell suspension and 2% w/v alginate were 1:1[13]. Then, the mixture was dripped to 0.1 M $CaCl_2$ using a syringe at ambient temperature[14]. The beads formed were let to solidify in $CaCl_2$ solution with a magnetic stirrer for 30 minutes[15]. The beads were then stored in the $CaCl_2$ solution for 16–120 hours at 4°C. Fermentation was carried out in batches with operating conditions and nutritional recipes and analysis of ethanol were the same as that of Kumoro et al. (2020)[16] The alginate beads were filtered and washed twice with excess distilled water before to use[17]. Cell growth in alginate beads before and after 48 hours of fermentation was cut into two parts previously and analyzed by SEM (Phenom ProX desktop SEM with EDX) at 2500 times magnification.

2.3 *Saccharomyces cerevisiae* (yeast) immobilization for removal of heavy metals from wastewater [6]

An effluent was obtained from one of the laboratories at the University of Passo Fundo, Brazil. A ratio of alginate solution (1 mol L^{-1} of phosphate buffer and 2% w/v of sodium alginate solution,) and yeast suspension (18% yeast suspension) was 1: 1. The mixture was stirred using Turax at 14,000 rpm for 10 minutes which was dropped onto 2% m/v $CaCl_2$. The formed beads were left for 2 hours at 4°C. The bead was washed with distilled water three times and dried 50°C for 24 hours before used. The beads were analyzed by Scanning Electron Microscopy (SEM). Heavy metal solutions were lead nitrate, Pb(II), potassium dichromate, Cr(VI), and cadmium nitrate, Cd(II). Meanwhile, the adsorption isotherm of models used in heavy metals was Langmuir, Freundlich, and Redlich-Peterson [6].

2.4 Immobilization of microalgae for removing potassium in vinasse[9]

Vinasse was obtained from the output of a distillation column in an industry in Brazil. Five hundred milliliters 500 mL of D. sub-spicatus (1.8 gL^{-1}) stock suspension was centrifuged for 20 minutes at 1844 g. The cell residue before use was resuspended in 50 mL deionized water, then diluted and mixed with 350 mL alginate solution, resulting in 400 mL alginate-microalgae solution (1, 2, or 3 g alginate 100 mL^{-1}). Visual observation of the characteristics of the beads in terms of their chemical stability and diameter. Meanwhile, vinasse as a microalgae growth medium was centrifuged at 1844 g for 20 minutes, pH 7.6, and 20°C. then the results are sterilized at a temperature of 121°C for 20 minutes. One hundred and twenty-five mL of Erlenmeyer flask containing 25 mL vinasse was added with several immobilized cells (total beads produced with 12.5 mL microalgae-alginate solution). Furthermore, the flask was stored under stirring at 100 rpm at 25°C and in dark conditions.

The bead with immobilized microalgae was produced following the same procedure as used for alginate solutions preparation by dissolving alginate 2% w/v in deionized water at 25°C. The mixture was stirred at 1000 rpm for 1 h (until homogeneous). Aliquots of the polymeric solution (12.5 mL) were dropped into 50 mL of a gently stirred 0.45 M $CaCl_2$ solution) using a needle attached to the tubing from the peristaltic pump using flow rate 10 $mL.min^{-1}$ and the separating distance from the needle tip and the surface of the $CaCl_2$ solution was set to about 10 cm. The beads were kept in $CaCl_2$ solution at 4°C for 3 hours and rinsed with deionized water. The shape and formation of the beads were then visually observed.

3. Results and Discussion

3.1 Production of biohydrogen by Reusing Immobilized Mixed Culture

Various forms of mixed culture immobilization bead with Alginate concentration of 2% in various appearances: at the beginning of extrusion (Figure 1), visual of reused bead on day 102 (Figure 3a) and its size (Figure 3b) with a calibrated sample scale (Figure 2), optical microscope (Figure 4). Meanwhile, the hydrogen produced from reused bead alginate is presented in Figure 5.

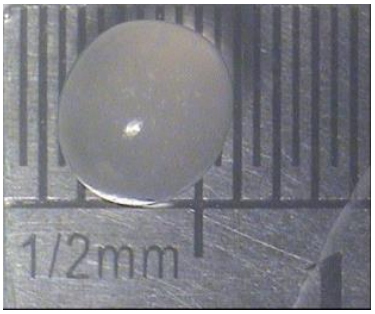

Figure 1. The physical appearance of synthesized initial alginate bead

Figure 1 shows that the alginate bead is ball-shaped and measures approximately 3.9 mm in diameter. This is consistent with the results of Al-Hajry *et al*. [18] which shows that the roundness of bead alginate greatly influences mechanical stability. Shapes and sizes can also be analyzed quantitatively using the Ohnesorge dimension (Oh) [19] where the influential parameters include the viscosity of the alginate solution, the dropper distance, and the stirring speed [20].

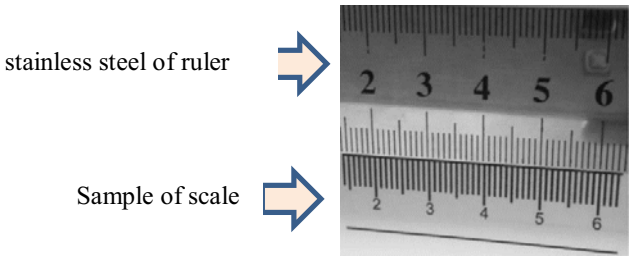

Figure 2. Sample scale calibration

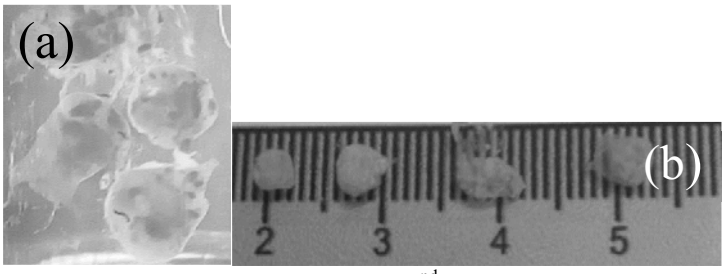

Figure 3. Visual of reused alginate beads on 102nd day (a) in distilled water (b) on scale

If the reused bead was used for 102 days, then the alginate starts to erode and transparent (Figure 3a) even though the size was still around 3.5 mm but the shape starts to flatten (Figure 3b). The erosion of the bead is believed to be due to the presence of calcium ions, which gradually dissolved causing the bead to be transparent, partially broken, and soft. Eventually, the beads become more fragile and destroyed [21].

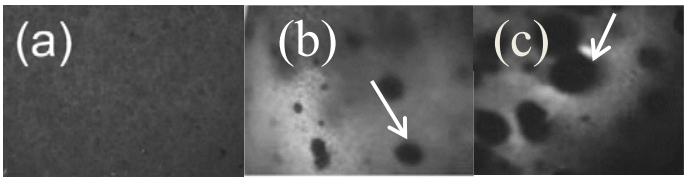

Figure 4. Growth of colonies on mixed culture immobilized beads observed using an optical microscope at 100× magnification on (a) day-0, (b) day-18, and at 40× magnification on (c) day-21.

Figures 4 depict that the longer the fermentation, the more microbial growth occurs in the bead. This is because glucose 2,845 nm (ChemSketch software) can pass through the pores of bead alginate which has a size of ~5nm [22]. The phenomenon of microbial growth followed by energy production in this application the same happens in hydrogen production [23].

Figure 5 shows that hydrogen in immobilized beads visually fluctuated for 34 cycles. These fluctuating hydrogen yields were similar to those obtained by Hu *et al.*[21] and Kumar *et al*. [24] show that the reused bead is made from 5% alginate with a crosslinking 0,1 M $CaCl_2$ solution able to produce stable production up to 60 days.

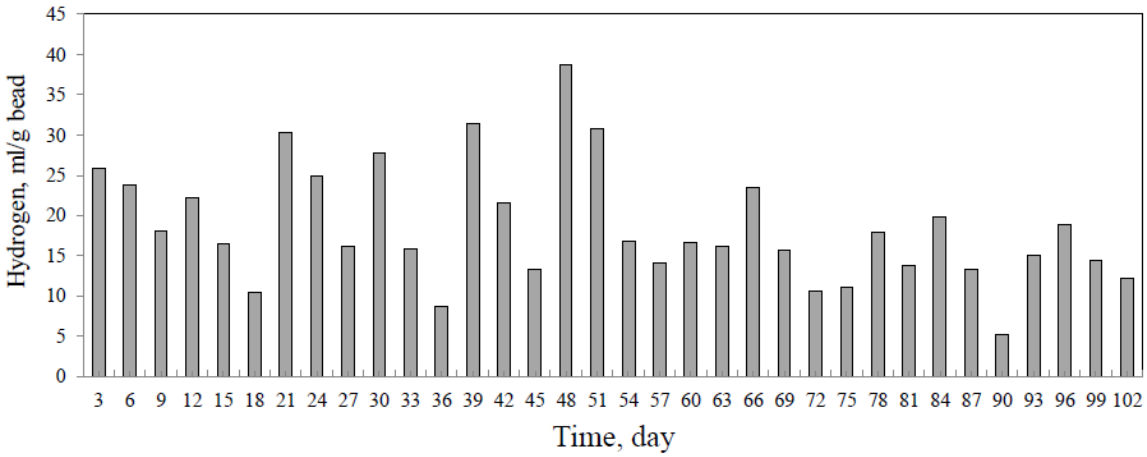

Figure 5. Biohydrogen production using immobilized mixed culture beads

Figure 5 also shows that the highest hydrogen yield is in the mid-cycle (16^{th}) of 38 $mL.g^{-1}$ beads. Repetition of reused bead was carried out every 3 days referring to previous studies [2], where the production of biohydrogen on the 3rd day had begun to decline. However, finally the longer the cycle, the hydrogen yield decreases (lower than the first three cycles). This shows that although the bead is increasingly eroded, because the substrate (glucose) and the buffer are always replaced every cycle, it means that microbes always get food supply and produce products. However, it turns out that microbes have a limited ability to produce or in other words microbes only maintain life. This phenomenon is under the microbial growth cycle which shows that the stationary phase occurs after the log phase [23]. The erosion of the bead can also cause a change in pH due to the loss of calcite ions in the bead so that hydrogen production is lower [21].

3.2 Bioethanol production using Immobilized Yeast

Visual observations of the immobilized yeast on alginate bead in various runs, microbial growth on the bead using SEM, and ethanol production are presented in Figure 6, Figure 7, and Figure 8.

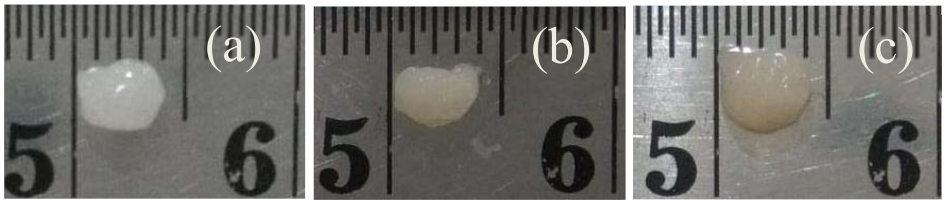

Figure 6. Bead ethanol production at (a) zero hours (b) 48 hours (c) reused 48 hours

Although the beads have a similar size which is around 4 mm, their shape is visually different (Figure 6). As seen in Figure 6 (a), Figure 6 (b), and Figure 6 (c), the bead is respectively rather round, not round, and almost perfectly round. However, the bead is shown in Figure 6 (c) looks flatter than the others. The phenomenon of the flat bead is also observed on alginate bead used in hydrogen production.

This indicates that the longer the bead is used, the more eroded the alginate bead will be due to the destability of alginate is influenced by cation affinity[25]. The short spots are seen in Figure 6 (a) are the

Saccharomyces cerevisiae yeast cells in the bead alginate at the beginning of the fermentation, whereas longer spots are observed in Figure 6 (b) as the *Saccharomyces cerevisiae* yeast cells in the bead alginate at 48 hours fermentation. As expected, more amount of yeast was found in Figure 6 (c) at longer fermentation time. This is thought to be due to fermentation that had been running before the bead was used again so that microbes have been able to adjust to their environment. This microbial growth is also following with the ethanol produced as is the case with hydrogen production.

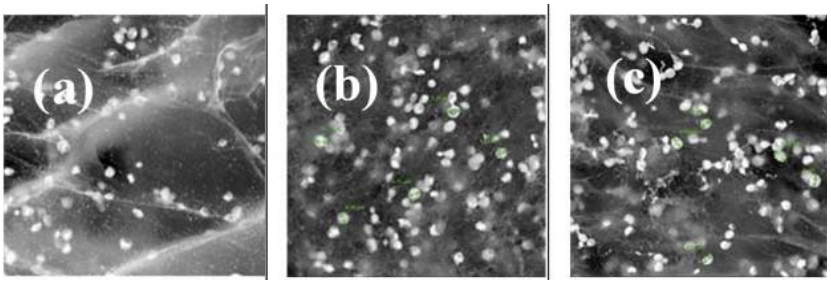

Figure 7. Morphology of S. *cerevisiae*-alginate bead: SEM at 2500× magnification: (a) initial (b) after 48 hours of fermentation (c) reused, 48 hours of fermentation [16]

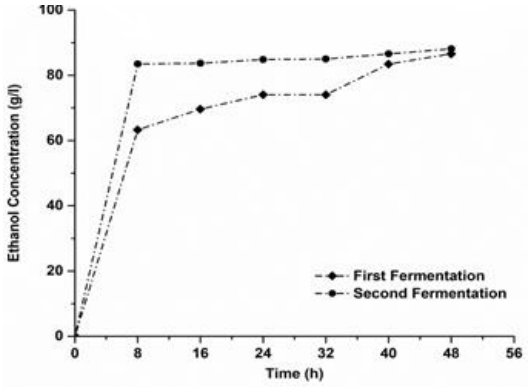

Figure 8. Production of ethanol using beads: initial (1st) and reused beads (2nd) fermentation cycle

Figure 8 shows that the maximum concentration of ethanol in the first and second fermentation cycle were respectively, 86.56 and 88.125 $g.L^{-1}$. This phenomenon is by previous research [26]. Ethanol concentration during the first, second, and third hours in the second fermentation cycle were 24%, 16%, and 12.7%, which were higher than that of the first fermentation cycle. This finding proved that the lag time used by immobilized yeast cells in the second fermentation cycle was very short because their number has reached a sufficient amount required by the system, while they have not achieved in the same period in the first fermentation cycle [27].

3.3 Yeast immobilization for removal of heavy metals from wastewater

The authors reported that the average size of the resulting bead was ± 1.81 mm with a spheroid shape with a smooth and porous surface. The spheroid shape of the beads is due to the drying of the alginate [28]. An

irregular internal structure of yeast beads and the presence of pores throughout their surface. The mechanism between adsorbent and adsorbate to predict adsorption capacity, which describes the relationship between the amount of adsorbate adsorbed on the adsorbent and its equilibrium concentration in aqueous solution, can be understood by the Langmuir and Freundlich isothermal model. These models are commonly used to explain heavy metal ion adsorption[29]. Biosorption isotherms for this study using Saccharomyces cerevisiae/alginate beads show based on the coefficient of determination (R2) of the largest order is Redlich and Peterson (0.969), Langmuir (0.965), and Freundlich (0.935). The biosorption isotherms also show that different biosorption capacities and affinities. The higher the biosorption affinity, the higher the adsorbed capacity. This is consistent with the research of Giles et al. (1960) [30] and Ma et al (2015)[31]. Whereas the maximum single layer removal affinities estimated by the Langmuir model were 179.1, 72.1, and 30.7 $mg.g^{-1}$ for Pb(II), Cr(VI), and Cd(II), respectively.

3.4 Immobilization of microalgae for removing potassium in vinasse

The authors reported that beads were spherical and the homogeneity was obtained with a concentration of 2% alginate and an average diameter of 0.92 cm. On the other hand, the lowest concentration of biopolymers (1%) resulted in brittle particles and defects and the average diameter was ±0.26 cm. This is following the research of Al-Hajry et al.,(1999)[18] and Damayanti et al. (2018)[32]. Meanwhile, the alginate beads (3%) were round and slightly flattened and the average diameter was ±0.53 cm. This is similar to the results of the study by Damayanti et al. (2018)[32] who used a binding agent of 0.1 M $CaCl_2$ and the average diameter was ±0.42 cm. The size and sphericity of the alginate particles affect their mechanical and chemical stability leading to limited diffusion rate and bead strength [20]. The cross-linked alginate beads with 2%, 5%, and 10% of $CaCl_2$ were able to remove 25%, 28%, and 35% potassium concentrations. This shows that cell immobilization in wastewater treatment has high efficiency [33][34]because the diffusion of calcium into alginate droplets is followed by diffusion of alginate molecules into the gel-forming zone causing high alginate concentrations at the interface. Ca^{+2} –alginate during alginate bead formation [35][36]. Thus, the higher the concentration of $CaCl_2$ (10%), the denser the Ca-alginate interface is to increase the uptake of potassium by the beads. Conversely, the low concentration of potassium cross-linked with 2% $CaCl_2$ is due to the high porosity of the matrix to diffuse nutrients.

4. Conclusion

Cell immobilization with alginate concentration of 2% w/v using dripping extrusion method produced sufficiently strong and homogeneous round shape beads suitable for various applications even though the concentration of $CaCl_2$ varies. The SEM image proved the occurrence of microbial growth during hydrogen and ethanol productions. The use of reused bead in maximum hydrogen production in the 16^{th} cycle, while ethanol production starts at the 8^{th} to 48^{th} hours. The SEM observations on yeast-alginate beads also demonstrated the presence of pores in alginate beads. In the equilibrium test, the highest order heavy metal removal affinities with the Langmuir model were Pb (II) 179.1 $mg.g^{-1}$, Cr (VI) 72.1 $mg.g^{-1}$, and Cd (II) 30.7 $mg.g^{-1}$. D. subspicatus immobilized alginate (2%) with varying concentrations of $CaCl_2$ (2, 5, and 10%) has a round shape and mechanical strength and most of the potassium from vinasse can be removed.

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