BIOLOGICAL HYDROGEN PRODUCTION VIA BACTERIA IMMOBILIZED IN CALCIUM ALGINATE GEL BEADS

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Abstract. Hydrogen can be biologically produced by fermenting sugars in a mixed bacterial culture under anaerobic conditions. However, the slow growth rate of hydrogen-producing bacteria limits the productivity of a suspended-growth reactor due to the requirement for long hydraulic resident time in order to maintain adequate bacteria population. Calcium alginate gel entrapment was studied in this research as a possible method for enhancing biomass density through bacteria immobilization. Sewage sludge was used as the source for the hydrogen-producing bacteria, after pretreatment using acid to eliminate the Methanogen archaea. Experimental results indicated that these hydrogen-producing bacteria maintained high activity within a range of pH, i.e., from 5 to 8. Calcium alginate gel beads effectively entrapped the hydrogen-producing bacteria, resulting in significantly increased hydrogen production rates. The immobilized hydrogen-producing bacteria with 30% inocula increased the hydrogen production over 50% when compared to the production from 15% inocula. Four repeated cultures were used to determine the effective life of the calcium alginate gel beads. The gel collapsed after 22 days, but during this time the hydrogen production held relatively constant. Cheese whey was used in this study as the nutrient for hydrogen production, and the results showed that dilution was required for the suspended fermentation to obtain maximum hydrogen production yield. However, for the immobilized hydrogen fermentation, undiluted raw cheese whey could be directly used to produce hydrogen with maximum yield probably because substrate inhibition was alleviated with the diffusion barrier provided by the immobilization matrix.

Keywords: biological hydrogen production; calcium alginate; immobilization; fermentation; cheese whey
INTRODUCTION

Hydrogen gas is a clean energy source, since it produces only water upon combustion, and it has the highest energy content per unit weight among any known fuels (Das and Veziroglu, 2001; Nath and Das, 2004). There are different ways to produce hydrogen, including electrolysis of water, thermo-catalytic reformation of hydrogen-rich organic compounds, and biological processes (Levin et al. 2004). Currently, hydrogen consumed as energy is equal to three percent of the world energy consumption and this number is expected to grow significantly in the future. However, hydrogen is currently mostly produced from non-renewable fossil fuels, which poses a problem for future energy security (Nath and Das, 2004).

Dark fermentative hydrogen production from sugar has attracted more and more attention in recent years because of its numerous advantages over other forms of hydrogen production. The most significant advantage of this process is that it requires no light energy. Additionally, it has no oxygen limitation problems because the process does not produce oxygen. Moreover, a variety of carbon sources can be used as substrates in the process, including negatively-valued raw materials such as sugar-containing wastewater (Nath and Das, 2004). Such hydrogen production via dark fermentation from various waste and wastewater sources has been reported, including sources such as bean curd manufacturing waste (Zhu et al. 2002), rice winery wastewater (Yu et al. 2002), starch wastewater (Zhang et al. 2003), livestock waste (Regan et al. 2004), olive oil waste (Eroglu et al. 2004), food processing, and domestic wastewater (Van Ginkel et al. 2005). Moreover, mixed culture has been widely utilized therefore requiring no sterilization in the process. For example, acclimated sewage sludge was cultured to generate hydrogen (Chen and Lin, 2001; Fang et al. 2002; Oh et al. 2003; Van Ginkel et al. 2001). Most studies for microbial hydrogen production used suspended-cell systems, which are normally inefficient or difficult to handle in continuous operations as a result of problems with recycling the biomass to maintain sufficient cell density in the reactor. Bacteria immobilization, however, which enhances the available bacteria population in the reactor, can increase fermentation rates, shorten the fermentation period, and increase the productivity. Recent studies show that different immobilization methods can be used as effective means to increase the hydrogen productivity (Wu et al. 2003) with both pure and mixed cultures. Examples include: agar gels-immobilizing *Rhodobacter sphaeroides* (Zhu et al. 1999); lignocellulosic materials supported *Enterobacter cloacae* (Kumar and Das, 2001); and ethylene-vinyl acetate copolymer immobilizing anaerobic sludge (Wu et al. 2005).
The immobilization techniques are based on the fact that bacteria can be entrapped within cross-linked polymers when a highly cross-linked network of polymers is formed in the presence of the bacteria. Such an immobilization method has a major advantage since it does not involve chemical modification of the bacteria. Alginate, a block copolymer of 1,4-linked $\beta$-D-mannuronic acid and $\alpha$-L-guluronic acid, can be a good polymer candidate for this application (Tan et al. 2003). It forms a calcium alginate gel when calcium ions are available. Entrapment by calcium alginate gel is widely used for immobilization of cells because it is a simple and reproducible technique using mild conditions (Shibasaki-Kitakawa et al. 2001).

An effective way for hydrogen fermentation cost reduction is the use of waste as feedstock. Cheese whey, a byproduct of the dairy industry, is the liquid remaining after the precipitation and removal of milk casein during cheese making. It represents about 85-90% of the milk volume and 55% of milk nutrients (Siso, 1996). The large quantity of nutrients in whey, including lactose, soluble proteins, lipids, and mineral salts, is not fully utilized because of the low value and limited market of whey products. Because of its low concentration of milk constituent (for example, lactose content is only 4.5-5% (w/v)), whey has been commonly considered as a waste product. Whey has high organic matter content, exhibiting a BOD$_5$ of 30g-50g/L and COD of 60-80g/L, with lactose being largely responsible for the high BOD and COD. Environmentally-friendly and economical disposal of these whey byproducts is a great challenge to the dairy industry (Liu et al. 2006). However, bioconversion of cheese whey to single cell protein, ethanol, and methane has been reported recently, presenting possible solutions (Audic et al. 2003; Demirel et al. 2005; Kelleher et al. 2002). Biological conversion of cheese whey to hydrogen, especially with immobilized fermentation of hydrogen, still needs to be investigated. In this study, hydrogen-producing bacteria were immobilized into calcium alginate gel beads, then, the immobilized bacteria were used to treat cheese whey and to produce hydrogen.

MATERIALS AND METHODS

H$_2$ PRODUCING SLUDGE

Anaerobic sewage sludge, taken from the Wastewater Treatment Plant in Pullman, WA, was pretreated using two methods: acid pretreatment (Chen et al. 2002) in which 100 mL of liquid sewage sludge was adjusted to pH 3.0 using 6 M HCl and shaken at 35°C for 24 hours; and heat pretreatment (Oh et al. 2003) in which 100 g of solid sewage sludge was heated at 100 °C in an oven for 2 hours. The acid-pretreated sewage sludge was then cultured in...
a continuously- stirred tank reactor (CSTR) with glucose as the medium using the hydraulic retention time of 12-18
hours at room temperature (pH at around 5.5). The fermentation broth in the CSTR was used as hydrogen-
producing seeds in the batch cultures.

**CALCIUM ALGINATE IMMOBILIZATION OF HYDROGEN PRODUCING BACTERIA**

A 2% (w/v) solution (solution I) was prepared by dissolving sodium alginate in the seed broth from the CSTR.
Solution I was then pressed through a nozzle into a beaker containing 2.5% (w/v) CaCl₂ solution (solution II) to
form the beads. Calcium alginate beads were allowed to harden for 12 hours in solution II, then the beads were taken
out, washed with water, and filtered. (Tan et al. 2003).

**CULTURE MEDIUM**

The glucose culture medium used for H₂ fermentation contained 20 g/L COD glucose (i.e., 18.75 g glucose /L.
Here COD is only from glucose and one mole glucose equals to 192 g COD) as the carbon source and sufficient
amounts of inorganic supplements (Wu et al. 2002), including: NH₄HCO₃ (5.24g/L), NaHCO₃ (6.72g/L), K₂HPO₄
(0.125g/L), MgCl₂ · 6H₂O (0.1g/L), MnSO₄ · 6H₂O (0.015g/L), FeSO₄ · 7H₂O (0.025g/L), CuSO₄ · 5H₂O
(0.005g/L), and CoCl₂ · 5H₂O (1.25 × 10⁻⁴g/L). The initial pH of the culture medium was about 8, and was adjusted
with 0.1M HCl and 0.1M NaOH at the experiments which measure the influence of initial pH on the hydrogen
production. The initial pH levels under investigation in this experiment range from 3 to 10. Cheese whey, from
Ferdinand’s Ice Cream, Pullman, WA, was diluted with water, pH adjusted to 7, and then taken directly as cheese
whey culture medium. The culture medium was stored at 4 °C in the refrigerator for no more than one week. Whey
used in this study had a COD of 70g/L, pH 6.5, and Total Ammonia Nitrogen (TAN) of 109.7mgN/L.

**BATCH CULTURE**

Twenty millimeters of culture medium were placed in a serum bottle. Three millimeters (15% inocula) or other
amount if mentioned specifically of hydrogen- producing seeds (fermentation broth from the CSTR or alginate
beads with the same amount of seed broth immobilized) was inoculated into the serum bottle for cultivation.
Nitrogen gas was pumped into the serum bottle for 2 minutes before fermentation to eliminate the oxygen inside the
bottle. The culture temperature was 35°C and the shaking speed was 100-150 rpm (Incubator IC 600, Yamato).

**REPEATED CULTURE**
After one batch culture of the immobilized bacteria, the calcium alginate beads were taken out, washed with water three times, and filtered. These beads were then put into a serum bottle with the same amount of original culture medium to start another batch that was cultured under the same conditions as described above. This process was repeated intermittently for 4 batches.

**SCANNING ELECTRON MICROSCOPY**

The surface morphology of the calcium alginate beads was examined using a scanning electron microscope (Hitachi S-570). Some of the freeze-dried beads were cut in half with a knife to inspect the structure inside. The gel beads, or their pieces, were mounted on metal stubs and their membranes were coated with gold for 6 minutes. Later, the surfaces were observed and photographed.

**ANALYSIS**

Total biogas production was measured by Owen’s method (Logan et al. 2002). The composition of biogas (H₂, CO₂, CH₄, and H₂S) in the headspace of the reactor was measured using a gas chromatograph (GC, CP-3800, Varian, Walnut Creek, CA) equipped with detectors, including: a thermal conductivity detector for H₂ and CO₂; a flame ionization detector for CH₄; a Valco Instrument Pulsed Discharge Detector run in Helium Ionization Mode D2 for H₂S; an 18” × 1/8” HayeSep Q 80/100 Mesh Silcosteel column for CO₂, H₂ and CH₄ with nitrogen as the carrier gas; and a 50m × 0.53mm × 4µm SilicaPLOT column for H₂S with helium as the carrier gas. The time to analyze one sample was about 8 minutes. A high-performance anion-exchange chromatography apparatus was used to analyze several salts of VFA, such as lactate, acetate, butyrate and propionate. The salts of VFA are analyzed using a Dionex DX-500 system (Sunnyvale, CA, USA) including an AS11-HC (4mm 10-32) column, a quaternary gradient pump (GP40), a CD20 conductivity detector, and an AS3500 auto-sampler. The eluent conditions are listed in Table 1 with the eluent flow rate: 1.5 mL/min; Pressure limit: 2000~3000psi; SRS: 100mA.

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Table 1. Eluent conditions for VFA analysis

a: Eluent A: 100 mM NaOH; Eluent B: 1mM NaOH; Eluent C: water.

5 RESULTS AND DISCUSSIONS

TREATMENT OF THE SEWAGE SLUDGE TO OBTAIN HYDROGEN-PRODUCING BACTERIA

As figure 1b illustrates, no methane was produced even after 6 days of suspended fermentation with the acid-treated sewage sludge. Very little methane was produced with the heat-treated suspended sewage sludge, although the amount increased with fermentation time. In comparison with the untreated sewage sludge, these results suggested that Methanogenic archaea, which consumes hydrogen to produce methane, was eliminated completely via acid-treatment of the sewage sludge. Most hydrogen-producing bacteria are spore-forming bacteria, forming spores when their environment is stressed. Heat treatment, which is designed to kill most of the competing microorganisms and promote the activity of the spore-forming hydrogen-producing bacteria, however, did not completely eliminate the methanogenic activity (fig. 1a, b), especially at long fermentation times. Although both acid- and heat-treatments have been reported to be effective ways to enrich hydrogen-producing bacteria (Chen et al. 2002; Oh et al. 2003), the results from this study indicated that the heat treatment used was not as effective as the acid treatment.
Figure 1a. Cumulative hydrogen production for different pretreatments of sewage sludge (Batch culture, 50ml glucose culture medium, initial pH=7.0). Data are the average of 4 duplicate tests.

Figure 1b. Cumulative methane production for different pretreatments of sewage sludge (Batch culture, 50ml glucose culture medium, initial pH=7.0). Data are the average of 4 duplicate tests.

THE INFLUENCE OF INITIAL pH ON HYDROGEN PRODUCTION

Figure 2a shows that the initial pH for the suspended culture medium was from 5.0 to 8.0. The hydrogen production did not show a significant change with the initial pH from 5 to 7, while the CO₂ / H₂ ratio increased
dramatically and hydrogen production decreased slightly above pH 7.0. Initial pH of the culture medium has been reported to play a very important role in biological hydrogen production. Van Ginkel et al. (2001) determined that the best pH for the hydrogen production was 5.5, while Zhang mentioned that the maximum hydrogen yield from starch wastewater was found at a wastewater pH of 6.0, and the maximum specific hydrogen production rate was at a pH of 7.0 (Zhang et al. 2003). There, the culture medium was glucose-liming medium with a large quantity of bicarbonate salt. As long as the fermentation produced by-products such as volatile fatty acids, they reacted with bicarbonate and the pH of the broth decreased with the release of the carbon dioxide gas. The CO₂ / H₂ ratio increased in the head space with an increase of the initial culture pH because greater pH reduction during the fermentation caused the subsequent release of more CO₂. Additionally, it has been reported that high CO₂ partial pressure directs the pathway away from hydrogen production (Park et al. 2005). Figure 2b shows that, at this metabolic shift at pH 8.0 and the higher CO₂ partial pressure, lactic acid production is seen to increase. Since lactic acid metabolism results in no hydrogen production, the hydrogen yield dropped slightly. In contrast to the effect of lactic acid, it seems that no significant influence on hydrogen production was observed by the acetate, butyrate, and propionate production. Lastly, as the initial pH went below 4 or above 9, the culture conditions appeared to be too harsh for the bacteria to grow and so little to no hydrogen or volatile fatty acid was produced.

![Figure 2a. Biogas production from the culture with different initial pH (batch culture, 20 ml glucose culture medium, 3 days, inocula 3ml). Data are the averages of triplicate tests with standard deviations (n=3) at α=0.05](image)
**BACTERIA IMMobilIZATION IN THE CALCIUM ALGINATE GEL BEADS**

The SEM picture clearly shows that hydrogen-producing bacteria (fig. 3b), mostly in the form of rod-like *Clostridium*, were entrapped within the 1.5mm diameter calcium alginate gel beads (fig. 3a). Due to the dehydrating effect of freeze-drying in order to prepare the sample for SEM, distortion of the gel beads in the form of surface wrinkles can be easily observed. A cryo-scanning electron microscope (cryo-SEM) has been used as an alternative for the structure analysis of the calcium alginate as it does not produce structure distortion as was seen in the SEM (Serp et al. 2002). Cryo-SEM studies have shown that polysaccharide chains inside the gel beads form in homogenously-distributed aggregates, giving rise to a network with a pore size under 100 nm (Serp et al. 2002). Nutrients diffuse into the gel beads and products spread out through these pores.
Figure 3a. SEM picture of surface structure of the calcium alginate bead, immobilized H₂ producing bacteria

Figure 3b. SEM picture of inside structure of the calcium alginate bead, immobilized H₂ producing bacteria

HYDROGEN PRODUCTION FROM BATCH CULTURE VIA IMMOBILIZED BACTERIA

Hydrogen production via immobilized bacteria reached its maximum in 2 days’ fermentation time while suspended culture needed 3 or more days (fig 4). As long as inocula for both suspended and immobilized culture were the same, there was no significant difference (p=0.097) in cumulative hydrogen production at the third day between suspended and immobilized culture, while hydrogen production from immobilized culture was higher than
hydrogen production from suspended culture at the second day \((p=0.004)\). This suggests that hydrogen production rates significantly increased due to bacteria immobilization.

![Graph showing cumulative H2 production](image)

**Figure 4.** Cumulative H2 production via immobilized and suspended culture (batch culture, 20ml glucose culture medium, initial pH=7.0, inocula 3ml, initial pH=8.0). Data are averages with error bars showing standard deviations \((n=3)\)

**THE INFLUENCE OF INOCULA SIZE ON HYDROGEN PRODUCTION**

For the suspended culture, hydrogen production increased with the increase in inocula in the first day of the fermentation, while no significant difference was observed in the second day of fermentation (fig. 5b). Inocula influenced the lag phase and production rate of the fermentation, but the cumulative hydrogen production ultimately was about the same. The suspended cell culture showed much higher hydrogen production at low levels of inocula (5%) compared to the production from immobilized cell culture at the second day of fermentation (fig. 5a, b).

Hydrogen production increased with the increase in inocula in both the first and second day of the immobilized cell culture, and the immobilized fermentation showed much higher hydrogen production at high levels of inocula compared to the production from suspended cell culture (fig. 5a, b). Immobilization inhibits cell reproduction and the cell growth rate remains low, which causes the yield to increase (Doran, 1995). In addition, large inocula shorten the lag phase for the fermentation, and higher inocula also shorten the hydraulic retention time, which favors the hydrogen-producing bacteria. When the inocula size reached 6ml, corresponding to 30% inocula for the culture, the
hydrogen production increased over 50% when compared to the production from 15% inocula of suspended cells (fig. 5a). Suspended-cell systems can hardly reach 15% inocula in a continuous production such as a CSTR system because “washout” may occur if the hydraulic retention time is too short. However, immobilized-cell systems can enhance the bacteria population, raising the inocula to as high as 30%, and increasing the productivity in the continuous operation.

Figure 5a. Cumulative H₂ production from immobilized cultures with different inocula (batch culture, 20ml glucose culture medium, initial pH=8.0). Data are averages with error bars showing standard deviations (n=3)
REPEATED CULTURE VIA IMMOBILIZED BACTERIA

The calcium alginate gel beads lasted for 22 days, then they all became transparent, soft, and partly broken, and were hard to filter and could not be collected after filtration. Four repeat cultures were carried out intermittently during this period and the hydrogen production in these repeat batches stayed relatively constant (fig. 6). Calcium alginate gel beads have been reported to be pH sensitive (Tan et al. 2003) and to undergo structure changes with the production of organic acids. Repeat acidic culture conditions lead to calcium loss and size erosion, which make the alginate beads fragile and finally collapse. Several approaches have been reported for enhancing the mechanical strength of calcium alginate beads, such as activated carbon addition (Wu et al. 2002).

Figure 5b. Cumulative H₂ production from suspended cultures with different inocula (batch culture, 20ml glucose culture medium, initial pH=8.0). Data are averages with error bars showing standard deviations (n=3).

Figure 6. Repeat H₂ production from repeat culture via immobilized bacteria (20 glucose culture medium, 3 days each batch, initial pH=8.0, inocula 3ml). Data are the average of duplicate tests.

BIOLOGICAL HYDROGEN PRODUCTION FROM CHEESE WHEY

As shown in figure 7a, for the suspended culture, when whey concentration was below 35g COD/L, hydrogen production from batch cultures increased with increase of whey concentration, that is, the amount of cheese whey
input into the culture medium, since the overall culture medium volume was a constant 20 ml in this study.

Hydrogen production remained constant or even slightly decreased when the whey concentration was passing over 35g COD/L to no dilution. This trend was clearer for the yield of hydrogen production, which reached a maximum 27ml hydrogen /g COD at whey concentration 35g COD/L and decreased with higher whey concentration. The same effect of substrate concentration on hydrogen production was also reported with sucrose, and hydrogen yield decreased as substrate concentration increased to the extent that substrate overload occurred (Kim et al. 2006; Kyazze et al. 2006). For the immobilized hydrogen fermentation (fig. 7b), hydrogen production from batch cultures increased with increased whey concentration while hydrogen yield remained constant. The entrapment of bacteria into the gel always creates mass transfer issues (Shibasaki-Kitakawa et al. 2001), and this mass transfer barrier can explain the difference in performance of hydrogen fermentation between suspended and immobilized culture because the substrate needs to diffuse into the gel to reach the bacteria. Raw cheese whey could be directly used to produce hydrogen with maximum yield because substrate inhibition was alleviated with the diffusion barrier provided by the immobilization matrix.

![Suspended Culture](image)

**Fig 7a.** Cumulative H2 production via suspended culture (batch culture, whey culture medium, initial pH=7.0, inocula 3ml, initial pH=8.0). Data are averages with error bars showing standard deviations (n=3)
Fig 7b. Cumulative H$_2$ production via immobilized culture (batch culture, whey culture medium, initial pH=7.0, inocula 3ml, initial pH=8.0). Data are averages with error bars showing standard deviations (n=3)

Summary

Sewage sludge was used as the bacteria source for the hydrogen production. Acid pretreatment of sewage sludge was proved to eliminate the Methanogen archaea and experimental results indicated that these hydrogen-producing bacteria maintained high activity within a pH range from 5 to 8. This study demonstrates an effective method to immobilize bacteria from sewage sludge, using calcium alginate entrapment, to produce hydrogen from glucose-limiting culture medium or cheese whey culture medium. Calcium alginate gel beads effectively entrapped the hydrogen-producing bacteria resulting in significantly increased hydrogen production rates. When cheese whey was used as a nutrient to produce hydrogen using suspended fermentation, dilution was required to obtain maximum hydrogen production yield. However, in the immobilized hydrogen fermentation process, undiluted raw cheese whey could be directly used to produce hydrogen with maximum yield because substrate inhibition was alleviated due to the diffusion barrier provided by the immobilization matrix. The hydrogen production reached 125 ml / g glucose or 23ml / g whey with immobilized fermentation, and the calcium alginate gel bead immobilization matrix was shown to last for 22 days until it collapsed. Further study is needed on increasing the yield of hydrogen from whey, and on increasing the effective life of the immobilization matrix.
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Literature Cited


