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Biohydrogen fermentation of galactose at various substrate concentrations in an immobilized system and its microbial correspondence

Periyasamy Sivagurunathan,[1,2] Arivalagan Pugazhendhi,[3] Gopalakrishnan Kumar,[4] Jong-Hun Park,[5] and Sang-Hyoun Kim[6,*]

Green Energy Technology Research Group, Ton Duc Thang University, Ho Chi Minh City, Viet Nam,[1] Faculty of Environment and Labour Safety, Ton Duc Thang University, Ho Chi Minh City, Viet Nam,[2] Green Processing, Bioremediation and Alternative Energies Research Group, Faculty of Environment and Labour Safety, Ton Duc Thang University, Ho Chi Minh City, Viet Nam,[3] Integrated Science and Engineering Division, Underwood International College, Yonsei University, Seoul 03722, Republic of Korea,[4] Department of Civil, Environmental and Architectural Engineering, Korea University, Seoul 02841, Republic of Korea,[5] and School of Civil and Environmental Engineering, Yonsei University, Seoul 03722, Republic of Korea[6]

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The effects of substrate concentration on fermentative hydrogen production from galactose at a fixed hydraulic retention time of 12 h were investigated in an immobilized continuously stirred tank reactor. Peak hydrogen production rate and hydrogen yield of 9.57 L/L-d and 1.10 mol/mol galactose$_{added}$, respectively, were obtained at a feed substrate concentration of 30 g/L and an organic loading rate of 60 L/L-d. Quantitative polymerase chain reaction analysis showed that the variations in the performance resulted primarily from metabolic alterations within the metabolism of the established microbial community rather than modifications in the population. The results obtained showed that optimal substrate concentration is essential for the efficient, continuous production of hydrogen from galactose.

[**Keywords:** Substrate concentration; Organic loading rate; Galactose; Hydrogen yield; Immobilization]

The role of energy in the progress of human civilization is quite remarkable. Indeed, energy production is a prerequisite of modern economy. Nevertheless, despite the technological advancements made, most energy is still derived from fossil fuels. Population growth rates indicate increased demand for food, fuel and employment, and reliance on fossil fuels to meet these requirements is creating environmental problems, by for example, increasing atmospheric CO_2 levels (1–3). Biomass transformation technology is being well used to produce biofuels like biodiesel, bioethanol, biobutanol and biogas. Presently, hydrogen is used only at the industrial level though it can be used as a means of generating electricity (4,5). Of the alternative technologies, fuel production from renewable feedstock has fewer greenhouse gas emissions from production to usage. Over the years, research has shown biohydrogen is a potential form of green-energy in terms of pollution control, production feasibility, and application practicalities (1).

Dark-fermentation and photo-fermentation are the two major approaches used by biohydrogen production systems. Dark fermentation is considered the most convenient way of producing biohydrogen from a variety of organic biomass using mixed cultures of microorganisms. However, the technology of biohydrogen production from biomass (i.e., hydrogen generation resulting from the degradation of organic matter by microorganism) is still in its infancy, although it has an eco-friendly signature due to the mild reaction conditions used, low pollutant discharge and energy consumption (6,7). Biofuels from biomass are described as second-generation biofuels, and are produced using lignocellulosic materials (e.g., agriculture waste) (2,8,9) in these feedstock, carbohydrates are the main ingredient responsible for fermentative biohydrogen production. Hence, biomass rich in carbohydrates with limited amounts of proteins and trace levels of lipids are best suited for biohydrogen production (10). Dark fermentation is performed under anoxic conditions and utilizes a wide variety of carbon sources from different substrates to produce valuable metabolites, such as, butyrate and acetate (10). Simple sugars, such as, glucose have also been shown to be potential substrates for pure cultures. Kim and co-workers (11,12) reported that galactose in marine algal biomass is an efficient substrate for hydrogen production. Galactose is the major monomeric sugar of several red algal species including *Gelidium amansii*, hence developing a hydrogen production from galactose substrate would helpful for harnessing the bioenergy recovery from algal biomass (13,14).

Recently, immobilization technologies, based on gel granulation and biofilm attachment processes, have been applied to hydrogen producing bioreactors to increase hydrogen yield (HY) and improve reactor stabilities. The immobilized cells also promote biofilm formation during prolonged operation in continuous stirred tank reactors (CSTRs), fixed bed reactors (FBRs), and upflow anaerobic sludge blanket reactors (UASBRs). Biofilm formation increases biomass contents and enables the use of high fluid velocities during wash cycles without removing cells (6,15–18). The most common matrix used for cell growth and biofilm attachment for hydrogen

* Corresponding author at: School of Civil and Environmental Engineering, Yonsei University, Seoul 03722, Republic of Korea. Tel.: +82 2 2123 2795; fax: +82 2 364 5300.
E-mail addresses: periyasamy.sivagurunathan@tdt.edu.vn (P. Sivagurunathan), sanghkim77@gmail.com (S.-H. Kim).

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production is activated carbon (AC), although fibrous support and cellulosic matrices are sometimes used. Cell entrapment in matrices allows high reactor accumulations of hydrogen producing bacteria, and thus, creates a local anaerobic environment to that promotes fermentative hydrogen production. Furthermore, hydrogen production rate (HPR) could be improved by increasing organic loading rates (19,20). The hydrogen production performance in mixed culture bioreactor would be manipulated by operating parameters such as organic loading rate (OLR) (20–24). Optimization of OLR would be achieved by the change of hydraulic retention time (HRT) (25). However, OLR is determined by not just HRT but also feed substrate concentration. In this research, the authors demonstrate the influence of feed substrate level on immobilized cell CSTR at a fixed HRT of 12 h. In addition, we examined microbial community population by quantitative polymerase chain reaction (qPCR).

MATERIALS AND METHODS

Inoculum source and the preparation of immobilized cells The anaerobic granular sludge inoculum used in this research was obtained from a local brewery wastewater treatment plant and had a pH of 7.7 and a total suspended solids (TSS) levels of 21.3 g/L. The inoculum conditioning and immobilized bead preparation was conducted as previously described (15). In brief, the granular sludge was heat-treated at 90 °C for 30 min in a water bath, dried in a hot-air oven at 100 °C overnight, and ground using mortar and pestle and obtained a uniform fine powder. This powder was then added to a solution of sodium alginate (2%), SiO_2 (1%) and chitosan (1%) for cell entrapment and encapsulation. The mixed suspension was extruded into a calcium chloride (2%) solution to form beads of uniform size (~6 mm). Galactose was used the carbon source at substrate concentrations from 20 to 40 g/L and mixed with basal modified Endo nutrient medium.

Immobilized bioreactor operation CSTR was used as an experiment bioreactor model and had an operating working volume of 3.0 L. The reactor temperature of 35 ± 1 °C was maintained using a thermal jacket layer and pH was maintained at over 5.5 by adding 3 N NaOH using a peristaltic pump. The stirrer was fixed on the top of the bioreactor and stirrer speed was set at 150 rpm to ensure uniform mixing. The CSTR was seeded with 500 g of immobilized cells and started in batch mode for 48 h before switching to continuous operation at a fixed HRT of 12 h. Initial substrate concentration was maintained at 20 g/L, and later increased to concentrations (25, 30, or 40 g/L) after the reactor had achieved pseudo-steady-state conditions.

Analytical methods The composition of the biogas (hydrogen, carbon dioxide, nitrogen, and methane) produced was determined by gas chromatography (model 310C, SRI Instruments, Torrance, CA, USA) using a thermal conductivity detector (TCD) (25). Biogas volume was measured using a wet gas meter (JH-LMF-1, Shanghai Jinghao International Trade Co., Ltd). Organic acids and galactose were quantified by high performance liquid chromatography (Waters 717, Waters, Milford, MA, USA) using an Aminex-87H column (Bio-Rad Laboratories, Hercules, CA, USA) and an ultraviolet detector (Waters 2487) at 210 nm and a refractive index detector (Waters 410). Procedures have been previously described in detail (11). Chemical oxygen demand (COD), pH, and TSS were measured using Standard Methods (26). Sigmaplot 10.0 and Microsoft-Excel 2013 were used for graphs drawing and data analysis.

Microbial community analysis Individual bacterial primer sets such as *Enterobacter*, *Enterococcus*, *Lactobacillus* sp., *Clostridium butyricum*, and total bacteria were used to quantify dominant bacterial populations in reactor effluent samples under various conditions. The detailed procedure used has been previously described (27). In brief, effluent samples from the bioreactor obtained at different times were subjected to DNA isolation, followed by qPCR (quantitative real time polymerase chain reaction analysis) using SYBR Premix Ex TaqTM (Takara Bio Inc., Shiga, Japan) in a BioRad CFX-96 real time system (Bio-Rad Laboratories). The oligonucleotide primer sets with specific annealing temperature (Ta) used were; Total bacteria (27-f 5′-AGAGTTTGATCMTGGCTCAG-3′, 518-r 5′-ATTACC GCGGCTGCTGG-3′) Ta (57 °C), *Enterobacter* sp. (EB-f 5′-GGACGGGTGAGTAATGTCT-3′, EB-r 5′-CTCAGACCAGCTAGGGATCG-3′) Ta (61.4 °C), *Enterococcus* sp. (EC-f 5′-TGGAAACAGGTGCTAATACCG-3′, EC-r 5′-GCTGCCTCCCGTAGGAGTC-3′) Ta (63.1 °C), *Lactobacillus* sp., (La-f 5′-GTGGGGGATAACATTTGGAA-3′Ta (59.3 °C), La-r 5′-GCCGATCAACCTCTCAGTTC-3′), and *Clostridium butyricum* (CB-f 5′CCTGCCTCATA GAGGGGAAT-3′, CB-r 5′-CGTGTCTCAGTCCCAATGTG-3′) Ta (59.3 °C). Each reaction mixture included 10 µl of SYBR Premix Ex Taq (TliRNaseH Plus, 2X), 0.4 µl of forward and of reverse primers (stock concentration, 10 µM), 0.4 µl of 50 × ROX Reference Dye II, 2 µl of template DNA, and sterilized distilled water to a final volume of 20 µl qPCR was performed using the following conditions: initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 10 s and primer annealing at specific primer Ta (°C) and extension for 34 s. Fluorescent signals were collected at the extension step. At the end of each run, a dissociation protocol (65 °C for 5 s, and 95 °C for 50 s) was performed to ensure the absence of nonspecific amplicons.

RESULTS AND DISCUSSION

Hydrogen production performances at different feed substrate levels The production performances obtained at various feed substrate concentrations are summarized in Fig. 1. Biogas production rates (BPR), HPR and HY were markedly influenced by variations in the organic loading rate. CSTR operated at a substrate concentration of 30 g/L and an organic loading rate of 60 g/L-d achieved maximal HPR and HY values of 9.57 L/L-d and 1.10 mol/mol $galactose_{added}$, respectively. HPR and HY decreased significantly on increasing the galactose concentration to 40 g/L. Previous reports have shown higher substrate concentrations enhance hydrogen production efficiency, but that this tends to peak (5,7). This deterioration in performance at higher substrate concentrations might be due to acid accumulation and increased hydrogen partial pressure (28). In this study also similar phenomenon occurred, where the increased substrate concentration results in lower hydrogen production efficiency, carbohydrate removal. Galactose removal efficiency was reduced from 91% to 87 % and HY also plummeted from 1.10 to 0.69 mol H_2/mol $galactose_{added}$. During the entire operation, the biogas was mainly composed of hydrogen and CO_2, while methane was not detected. Several technologies, such as scrubbing, pressure-swing adsorption and membranes are available for the separation of hydrogen from the H_2–CO_2 mixture in the full-scale (29). Interestingly, H_2 content increased on increasing substrate concentration from 20 to 40 g/L. After a drop-in hydrogen production at 40 g/L, the feed substrate concentration decreased back to 30 g/L during days 60–66 to evaluate the performance recovery. At the condition, HPR and HY were improved to 9.75 L/L-d and 1.15 mol/mol $galactose_{added}$, respectively. It meant that the effect of the overloaded substrate concentration was reversible for the H_2-producing microbiome (Table 1).

Table 2 summarizes the comparative assessment of the hydrogen production from galactose at various substrate concentrations (21,30–33). The maximum HPR of 9.57 L/L-d obtained from this study is comparable to previously reported literature values. Most of the studies employed a fair substrate concentration of 15 g/L under various HRT range from 8 to 24 h, the present study efficiently handles the high substrate concentration of 30 g/L at 12 h HRT and resulted in stable hydrogen production performances, however when the substrate concentration further exceeds to 40 g/L the remarkable drop in HPR and HY of 7.6 L/L-d and 0.68 mol/mol $galactose_{added}$ was noticed. The experimental evidence observed from this research suggests OLR plays an important role in hydrogen production and it has been previously suggested high OLR influencing the microbial dynamics, and the hydrogen production performances (19,34). Hence, maintaining a suitable substrate concentration is essential for the efficient, continuous production of hydrogen from galactose. Hydrogen production from marine algal source would be attractive and economically feasible while considering its full scale operation, which includes the extraction of hydrolysate, purification, operation and product purification are considered. Thus in our future study pilot scale operations and cost effective analysis would be added towards the development of sustainable BioH2 production from marine algal biomass.

Soluble metabolic acid production Organic acid production plays a major role in the generation of H_2, especially during mixed culture operation. Since the H_2 fermentation is a complex pathway

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