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# Changes in performance and bacterial communities in response to various process disturbances in a high-rate biohydrogen reactor fed with galactose

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## HIGHLIGHTS

- Self-aggregated biomass initiated by hybrid immobilization enabled $H_2$ production.
- The granular biomass was resistant to shorter HRT, extreme pH change, and starvation.
- Bacterial community principally consisted of *Clostridia*, *Bacilli*, and *Proteobacteria*.
- *Clostridia* were key bacteria in $H_2$ production using galactose as a carbon source.

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## ABSTRACT

High-rate biohydrogen production was achieved via hybrid immobilized cells fed with galactose in a continuous reactor system. The hybrid immobilized cells were broken down after 20 days and began to form granules by self-aggregation. The peak hydrogen production rate (HPR) and hydrogen yield (HY) of 11.8 ± 0.6 L $H_2$/L-d and 2.1 ± 0.1 mol $H_2$/mol galactose$_{added}$, respectively, were achieved at the hydraulic retention time (HRT) of 8 h with an organic loading rate (OLR) of 45 g/L-d. This is the highest yet reported for the employment of galactose in a continuous system. Various process disturbances including shock loading, acidification, alkalization and starvation were examined through bacterial community analysis via pyrosequencing of the 16S rRNA genes. The proportion of *Clostridia* increased during the stable biohydrogen production periods, while that of *Bacilli* increased when the reactor was disturbed. However, due to the stability of the self-aggregated granules, the process performance was regained within 4–7 days.



## 1. Introduction

The demand for energy has been rapidly increasing due to both technological advancements and increasing population. Until the last decade, fossil fuel reservoirs dominated the energy sector; however, their non-renewable nature and pollution issues and have recently led scientists to look for alternative clean energy and renewable sources (Demirbas, 2007; Long et al., 2013). In this spotlight, hydrogen, especially biohydrogen, has been considered as a promising energy carrier mainly because of its easy production methods, environmental friendly and clean nature, and applications in fuel cells (Das and Veziroğlu, 2001; Kumar and Lin, 2014; Lin et al., 2012).

The feedstock for production of biohydrogen varies widely depending on the region, ranging from wastewater to lignocellulose residue, and recently to algae biomass, especially marine algal biomass (Kumar et al., 2013; Park et al., 2013, 2011). One of the major issues with the employment of marine algal biomass is that the sugar component present is galactose, as its metabolic pathway is more complex than that of glucose. However, recent studies reported that galactose would be a feasible feedstock for dark fermentation for biohydrogen production (Park et al., 2011; Cheon and Kim, 2012; Kumar and Lin, 2014). Immobilization of the biomass has been reported as a suitable method for the packing of biohydrogen producing bacteria, since it could withstand washout at shorter hydraulic retention times (HRTs). Besides, immobilization aids in the self-aggregation process which results in granulation of the biomass, promising stable production and higher performance (Sivagurunathan et al., 2014a; Wu et al.,

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2005, 2006). The recently developed hybrid immobilization method was reported to be stable and reusable for many cycles (Sivagurunathan et al., 2014a).

Operation of a continuous stirred tank reactor (CSTR) is necessary for scaling up of the biohydrogen production process to the industrial level. The main parameter needing consideration in continuous operation is HRT, since it is vital for the stability of the biomass inside the reactor. At short HRT, the production performance increases, but biomass washout may occur (Hawkes et al., 2007; Wang and Wan, 2009; Wu et al., 2008). Furthermore, biohydrogen producing reactors are vulnerable to disturbance from various factors, including non-optimum organic loading rate, hydraulic retention times, pH, temperature, etc (Fang and Liu, 2002; Kim et al., 2006). Investigations of reactor operating conditions and organic acid content have been routinely conducted to pinpoint the parameters affecting the stable operation of reactors. Nevertheless, the reasons leading to failure of biohydrogen production are occasionally unclear. The microbial consortium constituting the reactor biomass needs to be identified to gain a better understanding of the biochemical reactions leading to the failure of biohydrogen production. Detailed information about composition of the microbial community together with conventional approaches would be helpful for analyzing biohydrogen production more systematically. Recent studies based on molecular techniques exploring microbial communities, such as high-throughput sequencing, quantitative-PCR, and fluorescent in situ hybridization, have shed light on disentangling the complex biochemical processes underlying biohydrogen production. However, the microbial communities in biohydrogen production reactors fed with galactose under various operating conditions have rarely been investigated.

Thus, the present study attempted to avoid the loss of biomass at short HRT through utilization of hybrid immobilized cells, and to induce the granulation process to attain higher biohydrogen production performance using galactose as a carbon source. Various process disturbances have occurred during CSTR operation. To the author's knowledge, this is the first report analyzing the production performances during various process disturbances. In addition, next generation sequencing techniques were used to reveal the microbial community structure with employment of various HRTs and process disturbances.

## 2. Methods

### 2.1. Feedstock and seed inoculum

The monosaccharide sugar galactose (Daejung, Korea) was used as the sole substrate. The seed sludge (anaerobic mixed cultures) was collected from an anaerobic digester at a local wastewater treatment plant to treat waste sludge. The characteristics of the sludge were as follows: total chemical oxygen demand (TCOD) 35.7 g COD/L, total suspended solids (TSS) 24.8 g/L, volatile suspended solids (VSS) 19.8 g/L, pH: 6.8, acetic acid 22.0 mg COD/L, propionic acid 11 mg COD/L, *n*-butyric acid 10.5 mg COD/L, lactic acid 10.1 mg COD/L. Heat treatment (90 °C for 30 mins in a water bath) was applied as pretreatment to cultivate/enrich spore-forming biohydrogen producers while also prohibiting the activity of hydrogen consumers (methanogens). The heat-treated sludge was dried and powdered using a grinder for use in the preparation of immobilized beads.

### 2.2. Fabrication of hybrid immobilized cells

The hybrid immobilized cells were prepared by the mixing of encapsulation and entrapment to ensure stability of the beads. Initially, 5% (w/v) of the dried inoculum was added to the prepared suspension of sodium-alginate (2%, w/v) solution, after which silicon dioxide ($SiO_2$ 1%, w/v) was added for mechanical strength and chitosan (1%, w/v) for entrapment and encapsulation of the biomass. Finally, activated carbon (2%, w/v) was added to provide the beads with porous nature, as mentioned in the previous study with slight modifications (Sivagurunathan et al., 2014a). The immobilization was carried out under a strict semi-anaerobic environment. The alginate-cell mixture was extruded into sterile calcium chloride solution (2%, w/v) for cell entrapment and formation of immobilized beads. The beads formed (5–6 mm) were further hardened by stirring in a fresh solution of calcium chloride for two more hours. Finally, the beads were washed three times with sterile distilled water and then dried before storage in the refrigerator until used in the experiments.

### 2.3. Continuous reactor set-up and operation

A CSTR with a working volume of 3.0 L (300 mm in liquid depth and 140 mm in inner diameter) was operated at a temperature of 35 ± 1 °C by feeding 15 g/L galactose according to the sugar concentration for a hydrolysate of red algal biomass (Park et al., 2011). The reactor was inoculated with 0.5 L of immobilized beads, and the pH value was controlled at 5.5 ± 0.1 by feeding 1 N NaOH (Kim et al., 2012). The HRT was varied from 12 to 8 h to increase the flowrate up to 150%. The operation period for each condition was more than 20 cycles at each HRT conditions, and pseudo-steady-state performance with stable biohydrogen production (±10% of produced hydrogen gas) was maintained for at least 3 consecutive days before the performance data were measured. During process disturbances the steady state was not attained, and the conditions were changed once the original performance of the early stage was retrieved.

### 2.4. Analytical procedure

$H_2$, $CO_2$, and other components in the biogas were analyzed by gas chromatography (GC, SRI 310, SRI Instrument) using a thermal conductivity detector as well as a 1.8 m × 3.2 mm stainless steel column packed with a mole sieve 5 A (SRI Instrument) and a 0.9 m × 3.2 mm stainless-steel column with a Porapak Q (80/100 mesh, SRI Instrument) (Kumar and Lin, 2014). Fermentation samples were collected on a daily basis and stored in the refrigerator at 4 °C prior to analysis of the parameters. For analysis of the volatile fatty acids (VFAs), alcohols, and sugars, 1 mL of the sample was centrifuged at 600*g* for 5 min and the supernatant was collected in a separate vial, which was used in a high-performance liquid chromatograph (HPLC, Waters) equipped with ultraviolet and refraction index detectors (Kumar and Lin, 2014). The limits for individual quantification of VFA, alcohol and sugar were 20 mg/L, 250 mg/L, and 250 mg/L, respectively. The standard methods of APHA were used for the analysis of COD and VSS (Park et al., 2014). The volume of the biogas produced was measured with a Mariotte device (water displacement method) and reported at STP (standard temperature and pressure). Sugar utilization rate was calculated based on the difference between the sugar remaining in the HPLC analysis and the initial sugar loaded into the reactor.

### 2.5. Bacterial community analysis

Biomass samples were collected at the startup period (P1, heat-treated inoculum) for the batch test, for the HRT of 12 h (P2, composite samples from day 14 to 17) and for the HRT of 8 h (P5, day 54–58), as well as just after shock loading (P3, day 20–24), acidification (P4, day 33–37), starvation (P6, day 61), and alkalization