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Fermentative Water Purification based on Bio-hydrogen

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생물학적 수소 발효를 통한 수처리 시스템

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Abstract

Among various techniques for hydrogen production from organic wastewater, a dark fermentation is considered to be the most feasible process due to the rapid hydrogen production rate. However, the main drawback of it is the low hydrogen production yield due to intermediate products such as organic acids. To improve the hydrogen production yield, a co-culture system of dark and photo fermentation bacteria was applied to this research. The maximum specific growth rate of *R. sphaeroides* was determined to be 2.93 h^{-1} when acetic acid was used as a carbon source. It was quite high compared to that of using a mixture of volatile fatty acids (VFAs). Acetic acid was the most attractive to the cell growth of *R. sphaeroides*, however, not less efficient in the hydrogen production. In the co-culture system with glucose, hydrogen could be steadily produced without any lag-phase. There were distinguishable inflection points in the accumulation of hydrogen production graph that resulted from the dynamic production of VFAs or consumption of it by the interaction between the dark and photo fermentation bacteria. Lastly, the hydrogen production rate of a repeated fed-batch run was 15.9 mL-H_2/L/h, which was achievable in the sustainable hydrogen production.

keywords : Bio-hydrogen, *Clostridium butyricum*, Co-culture, Dark fermentation, Photo fermentation, *Rhodobacter sphaeroides*

1. Introduction

The renewable energy is very important and essential in the view of social-economic and environmental issues. Due to its many advantages of no carbon dioxide emission and high energy density, hydrogen is found to be more environment friendly and a potential energy source compared to petroleum and other fossil fuels (Afgan and Carvalho, 2004). Among various techniques for hydrogen production, the biological fermentation is a feasible method since it can be carried out even in the ambient temperature and pressure using various organic compounds or wastes as an energy source (Demirel et al., 2010).

There are two ways of fermentation process capable of producing hydrogen (Kapdan and Kargi, 2006; Manish and Banerjee, 2008). One is a dark fermentation process where anaerobic bacteria such as *Clostridium* species produce hydrogen and various volatile fatty acids (VFAs) through acidogenic hydrogenesis (Brosseau and Zajic, 1982; Taguchi et al., 1995; van Andel et al., 1985). The other is a photo fermentation process using photosynthetic bacteria such as *Rhodobacter* or *Rhodopseudomonas* species, which enable to produce hydrogen from various VFAs at the expense of light energy (Barbosa et al., 2001; Fascetti et al., 1998; Shi and Yu, 2006). The structure of the dark fermentation system is very similar to a commercial anaerobic digestion process for the methane production, so it may be easy to attain commercial access by retrofitting conventional plant (Demirel et al., 2010). However, the oxygen sensitivity and low hydrogen production yield of the dark fermentation bacteria are known to be major drawbacks that have not been solved yet (Kraemer and Bagley, 2007). Theoretically, *Clostridium* species produce 4 moles of hydrogen from 1 mole of glucose with residual organic acids, which should be treated further to meet the affordable effluent quality. Compared with the dark fermentation bacteria, the photo fermentation bacteria has higher hydrogen yield and can utilize even VFAs as a sole carbon source (Fascetti et al., 1998). The limitation of not only substrate utilization but also low hydrogen production efficiency of a single stage of dark fermentation system would be eliminated. There-

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fore, some researchers have suggested a co-culture system of dark and photo fermentation bacteria (Odom and Wall, 1983; Yokoi et al., 1998). Higher hydrogen production yield can be obtained when dark and photo fermentative systems are combined. Further consumption of organic acids by the photo fermentation bacteria prevents pH drops in the fermenter due to the accumulation of fatty acids by dark fermentation bacteria. And the fermented liquid from the co-culture system satisfies the demand of intensified COD removal that is not met by the only single dark fermentation. In spite of their studies on the co-culture system, little information about pH and substrate is available in previous literatures.

Therefore, we examined the hydrogen production by pure- or co-culture of the dark and photo fermentation bacteria after the effects of carbon sources on the hydrogen production of each system were investigated in batch tests. The evaluation of a repeated fed-batch run for the co-culture system based on the continuous hydrogen production and the fermentation outlet composition was performed.

2. Material and Methods

2.1. Microorganisms and mediums

A dark fermentation bacterium, *Clostridium butyricum* and a photo fermentation bacterium, *Rhodobacter sphaeroides* were used as hydrogen-producing microorganisms in this study. Theses bacteria were purchased from the Korean Culture Center of Microorganisms (KCCM). *C. butyricum* was cultivated at 30°C for 7 h in the PYG medium (pH 6.5), which was composed of K_2HPO_4 (0.9 g/L), KH_2PO_4 (0.9 g/L), NaCl (0.9 g/L), $(NH_4)SO_4$ (0.9 g/L), $MgSO_4$ (0.09 g/L), $CaCl_2$ (0.09 g/L), peptone (10 g/L), yeast extract (5 g/L), cystein·HCl (0.5 g/L), $Na_2CO_3 \cdot 10H_2O$ (4.0 g/L), aminobenzoic acid (100 μL/L) and glucose (10 g/L). This medium was sterilized by autoclaving at 121°C for 15 min before being inoculated with *C. butyricum*. Purging the bioreactor with argon for 10 min could keep the culture system anaerobic.

R. sphaeroides was cultivated in a sterilized sistrom's minimal medium at 30°C for 72 h under tungsten lamps with 5,000 lux (Yokoi et al., 1998). Sistrom's minimal medium was composed of K_2HPO_4 (34.8 g/L) or KH_2PO_4 (27.2 g/L), $(NH_4)_2SO_4$ (5.0 g/L) or NH_4Cl (1.95 g/L), succinic acid (40.0 g/L), L-glutamic acid (1.0 g/L), L-aspartic acid (0.4 g/L), NaCl (5.0 g/L), nitrilotriacetic acid (2.0 g/L), $MgSO_4 \cdot 7H_2O$ (3.0 g/L) or $MgCl_2 \cdot 6H_2O$ (2.44 g/L), $CaCl_2 \cdot 2H_2O$ (0.334 g/L), $FeSO_4 \cdot 7H_2O$ (0.020 g/L), $(NH_4)_6Mo_7O_{24}$ (0.2 mL/L of a 1% solution), trace elements solution (1 mL/L) and vitamins solution (1 mL/L). The trace elements solution was prepared by adding 1.765 g EDTA, 10.95 g $ZnSO_4 \cdot 7H_2O$, 5.0 g $FeSO_4 \cdot 7H_2O$, 1.54 g $MnSO_4 \cdot H_2O$, 0.392 g $CuSO_4 \cdot 5H_2O$, 0.248 g $Co(NO_3)_2 \cdot 6H_2O$ and 0.114 g H_3BO_3 to a 100 m L of distilled water. After adding 1.0 g nicotinic acid, 0.5 g thiamine·HCl and 0.010 g biotin to a 1 L of distilled-deionized water, the vitamins solution was adjusted to pH 7.0. The mediums and solutions were stored at 4°C until being used.

2.2. Experimental procedure

A batch dark fermentation experiment was carried out in an acrylic reactor with 1 L working volume. Pre-cultivated *C. butyricum* was harvested after centrifugation at 4,000 rpm for 20 min and then re-suspended in the modified PYG medium (0.1 g dry cell/L). The modified medium was composed of 1.5 g/L KH_2PO_4, 4.2 g/L $Na_2HPO_4 \cdot 12H_2O$, 0.18 g/L $MgCl_2 \cdot 6H_2O$, 0.1 g/L $FeSO_4 \cdot 7H_2O$, 5 g/L peptone, 2 g/L yeast extract and 10 g/L glucose. Hydrogen evolved was collected using tedlar gasbags and the volume of gas was measured with an inverted cylinder containing a 10% NaOH solution.

Another reactor of 1 L working volume was used for a batch photo fermentation experiment. The pre-cultured *R. sphaeroides* was inoculated in the modified sistrom's minimal medium, where multi-organic acids of acetate, propionate, butyrate and lactate replaced succinic acid as a carbon source were input. Glutamic acid of 10 mM was also added instead of $(NH_4)_2SO_4$ as a nitrogen source. Different pH runs with 6.25 and 7.0 were applied to find the practical operation condition for a single culture of *R. sphaeroides*. And then acetate or mixed acid (synthetic acid or fermented liquid from dark fermentation with glucose) was used as a sole carbon source for the photo fermentation in order to investigate the effects of various substrates on the hydrogen production. The photo fermentation experiment was carried out under an illumination of 5000 lux and 30°C after the replacement of gas phase in the reactor with argon (Yokoi et al., 1998).

A co-culture of *C. butyricum* and *R. sphaeroides* was accomplished by using the modified PYG medium containg 10 g/L glucose as a sole carbon source in the 1 L acrylic reactor. A repeated fed-batch run of the co-culture system (pH 6.25) was conducted at 30°C and 5000 lux of light intensity.

2.3. Analytical methods

The hydrogen in the evolved biogas was analyzed using a gas chromatography (Agilent 7890, USA) equipped with a capillary column (Agilent 19095P-MS6. 300°C, 30 m, 535 μm, 25 μm) and a thermal conductivity detector (TCD). The operation temperature of an oven, injector and detector

was 50, 200 and 250°C, respectively. Helium was used as a carrier gas with a flow rate of 11.15 mL/min.

Volatile fatty acids (VFAs) in the fermentation effluents were also measured by the gas chromatography equipped with a capillary column (Restek 922303 260°C, 30 m, 530 μm, 0.25 μm) and a flame ionization detector (FID). Helium gas flowed with a splitting ratio of 10 to 1 as a carrier gas. The temperature of the oven was increased up to 145°C with a rate of 20°C/min thereafter maintained at 95°C for 2 min. and then increased again up to 200°C with a rate of 50°C/min. Injector and detector were set at 200 and 240°C, respectively.

Glucose concentration was detected by the 3,5-dinitrosalicylic acid (DNS) method according to the previous literature (Alalayah et al., 2008). The cell concentration in the medium was determined as suspended solid (SS) according to the Standard Methods (APHA, 1998).

3. Results and discussion

3.1. Effect of carbon source on photo fermentation process

Besides glucose, the photo fermentation bacteria are known to utilize VFAs as a carbon source as well (Fang et al., 2006). Thus, a mixed acid-utilizing activity of *R. sphaeroides* was examined and compared with an acetate-utilizing activity of it. Fig. 1. illustrates some factors to be considered when choosing a carbon source for the hydrogen production by *R. sphaeroides*. The maximum specific growth rates of this photo-fermentation bacterium were determined to be 0.25 and 2.93 h^{-1} when using 6 g/L VFAs and 3 g/L acetic acid, respectively. It implied that acetic acid was most attractive to the cell growth of *R. sphaeroides*. If considering the hydrogen production, however, the single substrate of acetic acid was not moderate due to the lower

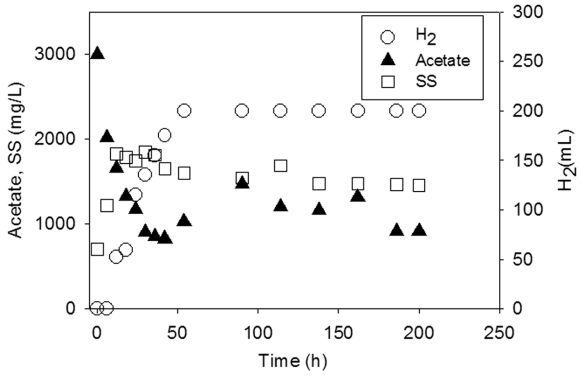


Fig. 2. Time-dependent SS, acetate and accumulated amount of hydrogen in a pure-culture system of *R. sphaeroides.*

hydrogen production yield in this study. From the viewpoint of hydrogen production, *R. sphaeroides* could produce more amount of hydrogen from VFAs with the higher production rate of 1.48 mL-H_2/L/h and 92.6 mL-H_2/g COD. When the mixture of VFAs was used as carbon source, *R. sphaeroides* quickly grew via the utilization of acetic acid and produced hydrogen using various VFAs as electron donors, especially lactic acid.

As can be seen in Fig. 2, *R. sphaeroides* grew fast with a little consumption of glucose for 12 h and then produced hydrogen up to about 200 mL. When acetic acid was used as a sole carbon source for the photo fermentation, *R. sphaeroides* successfully used it for their growth as well as the hydrogen production. However, about 900 mg/L of acetic acid remained in the medium. Acetic acid was efficient for *R. sphaeroides* to use for their growth rather than for the hydrogen production. A similar result had been reported in the literature (Fang et al., 2006).

The performance of hydrogen production based on the utilization of a single substrate and mixed acid was evaluated (Fig. 3). The hydrogen evolved with the mixed acid

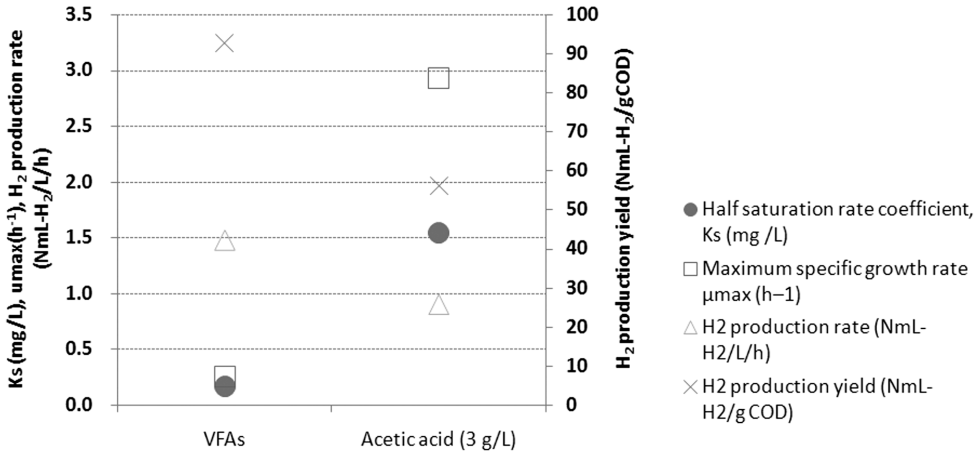


Fig. 1. Cell growth and hydrogen production coefficients of *R. sphaeroides* according to the types of carbon source.
[a] It was composed of 2 g/L acetic acid, 1 g/L butyric acid, 2 g/L lactic acid and 1 g/L propionic acid

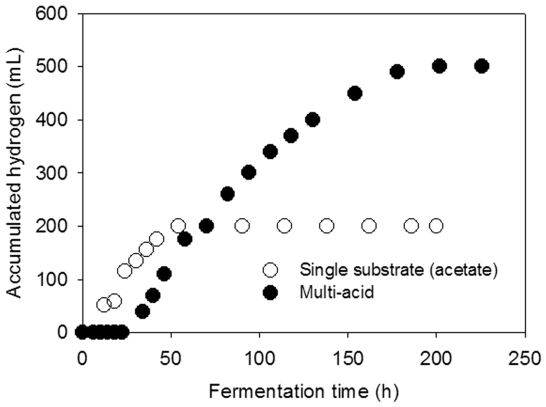


Fig. 3. Time-dependent accumulated amount of hydrogen with different substrates in a pure-culture system of *R. sphaeroides*.

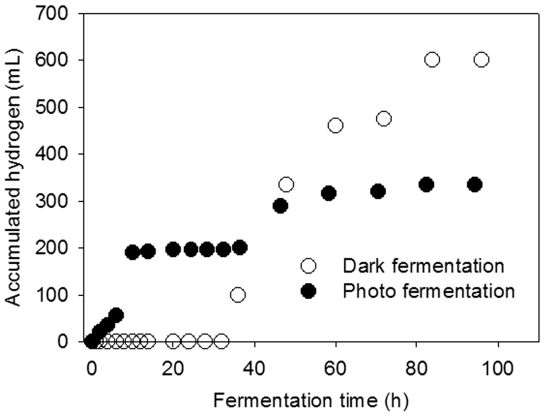


Fig. 4. Time-dependent accumulated amount of hydrogen in a two-phase system of *C. butyricum* and *R. sphaeroides*.

reached up to 500 mL, which was more than double that of using substrate. However, the lag phase for hydrogen evolution when acetate was fermented was quite short with only 6 h. It was thought that an adaption time was likely to be needed for *R. sphaeroides* to consume the mixed substrate. Time-dependent accumulated amount of hydrogen in a two-phase system of *C. butyricum* and *R. sphaeroides* is shown in Fig. 4. A photo fermentation process using photosynthetic bacteria such as *Rhodobacter* enables to produce more hydrogen from various VFAs, which is usually provided by the dark fermentation bacteria. On this study, the effluent from the dark fermentation of glucose by a pure culture of *C. butyricum* was fed to a consecutive photo fermentation system. Total 935 mL of hydrogen evolved was observed in the two-phase system. There was a quite long period of lag phase for *C. butyricum* to produce hydrogen while the photo fermentative bacteria could produce hydrogen at a very short adaption time with only 2 h. Besides the dark fermentation, nearly 50% additional hydrogen was evolved through the two-phase system.

3.2. Hydrogen production in a co-culture system of *C. butyricum* and *R. sphaeroides*

A co-culture system of *C. butyricum* and *R. sphaeroides* was performed with 10 g/L glucose as a sole carbon source. As shown in Fig. 5, hydrogen could be rapidly produced in proportion to the depletion of glucose in the co-culture system. There was a distinguishable inflection point in the accumulated amount of hydrogen evolved. The hydrogen production rate was very fast up to 19 h after that decreased significantly. It resulted from not only the different utilization time of glucose but also the dynamic production and consumption of VFAs by both the dark and photo fermentation bacteria.

Fig. 6 also shows dynamic changes of VFAs concentration in the co-culture system. Lactic acid was produced in the initial stages but disappeared in the end. It clearly proved that lactic acid was most preferable to the hydrogen production by the photo fermentation bacterium, *R. sphaeroides*. The concentrations of other VFAs, such as acetic acid, butyric acid and propionic acid, steadily increased over the entire period of fermenter run. Especially, acetic acid was largely generated even after glucose was used up. In addition,

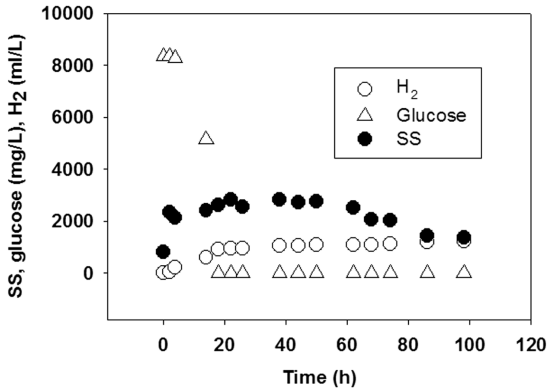


Fig. 5. Time-dependent concentrations of SS, glucose, and accumulated amount of hydrogen evolved in a co-culture system of *C. butyricum* and *R. sphaeroides*.

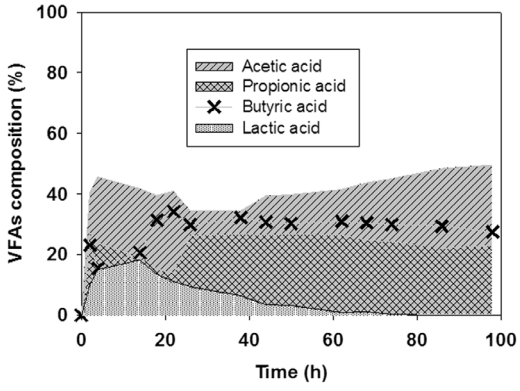
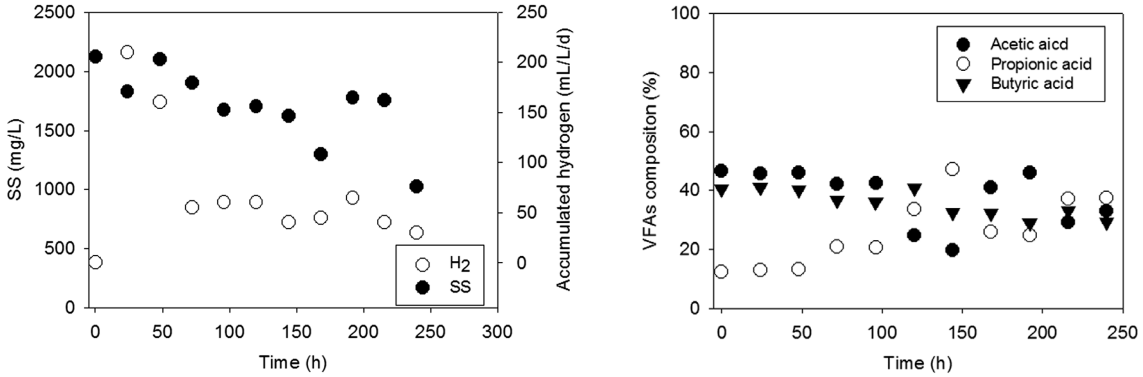


Fig. 6. Time-dependent composition of VFAs in a co-culture system of *C. butyricum* and *R. sphaeroides*.



(a) SS (b) VFAs

Fig. 7. Time-dependent concentrations of SS, composition of VFAs as well as daily accumulated amount of hydrogen evolved by the repeated fed-batch operation of the co-culture system.

the concentrations of propionic acid and lactic acid were higher than those in the pure culture system. These results implied that the co-fermentation system derived the production of another intermediates from glucose and resulted in changes in the concentrations of VFAs. The production rate of hydrogen by the co-culture system was determined to be 12.4 mL-H_2/L/h.

The co-culture system of *C. butyricum* and *R. sphaeroides* was also operated in the repeated fed-batch run, where 200 mL of the culture broth was replaced with the same volume of fresh medium everyday. Fig. 7 shows the time-dependent concentration of SS, compositions of VFA and accumulated amount of hydrogen evolved. The hydrogen production was stabilized in 3 d and its daily production was determined to be 77 mL/d. The hydrogen production rate of the repeated fed-batch run was 15.9 mL-H_2/L/h, which implied that the sustainable hydrogen production could be achieved in the co-culture system. Residual glucose and lactic acid were not detected over the entire period of test. It meant that the easy-degradable carbon sources were completely used as carbon sources in the co-culture system.

4. Conclusions

The maximum specific growth rate of this photo fermentation bacterium was determined to be 2.93 h^{-1} when using 3 g/L acetic acid. This implied that acetic acid was most attractive to the cell growth of *R. sphaeroides*. However, the single substrate of acetic acid was not available due to the lowest hydrogen production yield compared to a mixture of VFAs (acetic acid, butyric acid, lactic acid and propionic acid). *R. sphaeroides* quickly grew with acetic acid and produced a considerable hydrogen (92.6 NmL-H_2/g COD) using various VFAs as electron donors when the mixture of VFAs was used as carbon source.

In the co-culture system of *C. butyricum* and *R. sphaeroides*, hydrogen could be steadily evolved without any lag-phase with a marked inflection point on the time-dependant graph of the accumulated hydrogen evolved. This was the result from the dynamic production of VFAs by the dark fermentation bacteria and the simultaneous consumption of VFAs produced by the photo fermentation bacteria. Lastly, the repeated fed-batch run of co-culture system clearly showed the possibility of the stable hydrogen production of 15.9 mL-H_2/L/h. Higher and sustainable hydrogen production yield could be obtained due to the additional consumption of byproducts from the dark fermentation when dark and photo fermentative systems was combined. Thus, it is expected that the application of the photo fermentation bacteria on a conventional fermenter will be an affordable water-energy nexus facility in the view of reduction of the organic pollutant load and enhanced biohydrogen production.

국문요약

유기성폐수의 혐기발효 공정은 빠른 수소생성속도를 나타내며, 동시에 수중의 유기물을 처리한다. 반면, 수소생성 수율이 낮고 처리 수 내 혐기발효 산물인 복합 유기산이 다량 존재하게 된다. 따라서, 본 실험에서는 수소생성 수율을 높이고 처리수의 수질 제고를 위해 광발효미생물을 이용하였다. 광발효미생물의 기질에 따른 수소생산 속도 및 미생물 성장율을 조사하기 위해 아세트산, 복합 유기산 (인공) 및 글루코스 대상 혐기발효 상등액을 각각 기질로 이용하는 회분식 실험을 실시하였다. 아세트산을 이용한 *R. sphaeroides* 의 최대 비증식속도는 2.93 h로서 복합유기산을 이용할 때보다 높았다. 아세트산은 미생물 증식에 유리한 기질인 반면, 수소생산속도 면에서는 복합유기산보다 느리게 나타났다. 글루코스 혐기 발효액 상등액을 기질로 이용한 광발효에서 전단의 혐기발효를 통한 수소생산량의 약 50%가 추가로 발생하였다. 혐기 및 광발효미생물의 혼합발효 연속시스템을 통해 15.9 mL-H_2/L의 안정적인 수소를 생산하였다.

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References

Afgan, N. H. and Carvalho, M. G. (2004). Sustainability assessment of hydrogen energy systems. *Int. J. Hydrog. Energy*, **29**(13), pp. 1327-1342.

Alalayah, W. M., Kalil, M. S., Kadhum, A. A. H., Jahim, J. M., and Alauj, N. M. (2008). Hydrogen production using *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564). *Int. J. Hydrog. Energy*, **33**(24), pp. 7392-7396.

APHA (1998). Standard Methods for the Examination of Water and Wastewater, 20th ed., American Public Health Association, Washington, DC.

Barbosa, M. J., Rocha, J. M. S., Tramper, J., and Wifjjels, R. H. (2001). Acetate as a carbon source for hydrogen production by photosynthetic bacteria. *J. Biotechnol.*, **85**(1), pp. 25-33.

Brosseau, J. D. and Zajic, J. E. (1982). Hydrogen-gas production with *Citrobacter intermedius* and *Clostridium pasteurianum. J. Chem. Technol. Biot.*, **32**(3), pp. 496-502.

Demirel, B., Scherer, P., Yenigün, O., and Onay, T. T. (2010). Production of methane and hydrogen from biomass through conventional and high-rate anaerobic digestion processes. *Cri. Rev. Env. Sci. Tec.*, **40**(2), pp. 116-146.

Fang, H. H. P., Zhu, H., and Zhang, T. (2006). Phototrophic hydrogen production from glucose by pure and co-cultures of *Clostridium butyricum* and *Rhodobbacter sphaeroides*. *Int. J. Hydrog. Energy*, **31**(15), pp. 2223-2230.

Fascetti, E., D'Addario, E. Todini, O., and Robertiello, A. (1998). Photosynthetic hydrogen evolution with volatile organic acids derived from the fermentation of source selected municipal solid wastes. *Int. J. Hydrog. Energy*, **23**(9), pp. 753-760.

Kapdan, I. K. and Kargi, F. (2006). Bio-hydrogen production from waste materials. *Enzym. Microb. Technol.*, **38**(5), pp. 569-582.

Kraemer, J. T. and Bagley, D. M. (2007). Improving the yield from fermentative hydrogen production. *Biotechnol. Lett.*, **29**(5), pp. 685-695.

Manish, S. and Banerjee, R. (2008). Comparison of biohydrogen production processes. *Int. J. Hydrog. Energy*, **33**(1), pp. 279-286.

Odom, J. M. and Wall, J. D. (1983). Photoproduction of H_2 from cellulose by an anaerobic bacterial coculture. *Appl. Environ. Microbiol.*, **45**(4), pp. 1300-1305.

Shi, X. Y. and Yu, H. Q. (2006). Continuous production of hydrogen from mixed volatile fatty acids with *Rhodopseudomonas capsulata, Int. J. Hydrog. Energy*, **31**(12), pp. 1641-1647.

Taguchi, F., Mizukami, N. Saito-Taki, T., and Hasegawa, K. (1995). Hydrogen production from continuous fermentation of xylose during growth of *Clostridium* sp. strain No.2. *Can. J. Microbiol.*, **41**(6), pp. 536-540.

van Andel, J. G., Zoutberg, G. R., Crabbendam, P. M., and Breure, A. M. (1985). Glucose fermentation by *Clostridium butyricum* grown under a self generated gas atmosphere in chemostat culture. *Appl. Microbiol. Biotechnol.*, **23**(1), pp. 21-26.

Yokoi, H., Mori, S., Hirose, J., Hayashi, S., and Takasaki, Y. (1998). H_2 production from starch by a mixed culture of *Clostridium butyricum* and *Rhodobacter* sp. M-19. *Biotechnol. Lett.*, **20**(9), pp. 895-899.