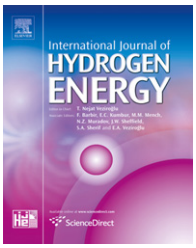
Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/he



Bio-hydrogen production by mixed culture of photo- and dark-fermentation bacteria

Bing-Feng Liu, Nan-Qi Ren, Jing Tang, Jie Ding, Wen-Zong Liu, Ji-Fei Xu, Guang-Li Cao, Wan-Qian Guo, Guo-Jun Xie*

State Key Lab of Urban Water Resource and Environment (SKLUWRE), School of Municipal and Environmental Engineering, Harbin Institute of Technology, No. 202 Haihe Road, 2nd Campus of HIT Box 2614, Harbin 150090, China

ARTICLE INFO

Article history:
Received 8 April 2009
Accepted 5 May 2009
Available online 6 June 2009

Keywords:
Bio-hydrogen
Clostridium butyricum
R. faecalis
Mixed culture

ABSTRACT

Clostridium butyricum and *Rhodopseudomonas faecalis* RLD-53 were employed to produce hydrogen in mixed culture with glucose as sole substrate. Due to the great difference on growth rate and acid-resistant capacity between photo-fermentative bacteria and dark-fermentative bacteria, directly mixed culture of the two kinds of bacteria in different ratio was studied in this work. Hydrogen yield, volatile acids, pH and biomass in different periods were evaluated. Acetic acid and butyric acid produced by *C. butyricum* were dominant terminal fermentation products, and they were effective substrates for photo-fermentative bacteria. The cooperation was formed in a way like food chain. But compared to the production rate of volatile acids produced by *C. butyricum*, the utilization rate by photo-fermentative bacteria was far slower. The results demonstrated that the growth of photo-fermentative bacteria was limited when pH decreased sharply. The best ratio of *C. butyricum* to *R. faecalis* RLD-53 was 1:600. The maximum yield of hydrogen reached 122.4 ml-H_2/vessel and hydrogen production rate was 0.5 ml-H_2/ml-culture/day.

1. Introduction

Hydrogen is acknowledged as an ideal clean energy carrier in 21st century. Dark-fermentation bacteria, photo-fermentation bacteria and algae are main functional microorganisms in hydrogen production. Volatile organic acids (VFAs) are generated from large molecular substrates by dark-fermentation, such as acetic acid, propionic acid and butyric acid. Those acids lead to a sharp decrease of pH and H_2 production was limited. However, photo-fermentation bacteria can further use VFAs to produce H_2. So mixed culture of photo- and dark-fermentation bacteria has been concerned by researchers for a high efficiency hydrogen production.

The approach of mixed-culture pattern has been proved to be feasible. And at the same time, it is found that the cooperation of the two kinds of functional bacteria can bring out a higher production yield and energy needed by photosynthetic bacteria is saved in the process. Weetall et al. [1] used agar immobilization of *Rhodospirillum rubrum* and *Klebsiella pneumoniae* in mixed way to produce hydrogen from cellulose and max hydrogen yield reached 6 mol-H_2/mol-glucose. Odom and Wall [2] mixed *Rhodopseudomonas capsulata* and *Cellulomonas*. sp to utilize cellulose for hydrogen and the result showed 4.6–6.2 mol-H_2/mol-glucose. Miyake et al. [3] increased hydrogen yield from 1.1 mol-H_2/mol-glucose to 7 mol-H_2/mol-glucose by combination of photosynthetic

* *Corresponding author*. Tel.: +86 451 86282008.
E-mail address: rnq@hit.edu.cn (N.-Q. Ren).

0360-3199/$ – see front matter © 2009 Professor T. Nejat Veziroglu. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijhydene.2009.05.005

bacteria and *Clostridium butyricum*. Yokoi et al. [4] got the level of 6.6 mol-H_2/mol-glucose by mixture of *C. butyricum* and *Rhodobacter* sp. M-19, and it was concluded four times higher than single anaerobic bacteria. Because of the different properties between the two kinds of bacteria, it was hard to coexist and difficult to study further. In recent years, Asada et al. [5] immobilized *Lactobacillus* and *Rhodobacter sphaeroides* RV together for hydrogen from glucose and the results were 7.1 mol-H_2/mol-glucose. Herbert H.P. Fang et al. [6] mixed *C. butyricum* and *R. sphaeroides* to produce hydrogen and made quantitative analysis of two microbial communities in FISH technology. In a conclusion, the mixed culture of photo- and dark-fermentation bacteria can improve utilization rate of substrate and enhance hydrogen yield. But the further studies focus on how to make bacteria in good cooperation and how to control the substrate conversion in a food chain.

In this study, a mixed-culture pattern was investigated in different ratios of *C. butyricum* and *Rhodopseudomonas faecalis* RLD-53. Glucose was used as the sole substrate for hydrogen production. The hydrogen yield, volatile acids, pH of system and biomass were determined. It is expected that the results of pilot studies obtained from this study could provide useful information for further mixed-culture hydrogen production.

2. Material and methods

2.1. Bacterium and growth conditions

The photo-fermentation bacterium, *R. faecalis* RLD-53, was isolated from freshwater pond sludge [7]. The previously described medium [8] was used as the medium for pre-culture of photo-fermentation bacterium (PFBM).

The dark-fermentation bacterium, *C. butyricum*, was purchased from China General Microbiological Culture Collection Center, AS 1.209. The medium for pre-culture for Dark-fermentation bacterium (DFBM) consists of per l.0 L, glucose 9 g, $(NH_4)_2SO_4$ 2 g, yeast extract 1 g, K_2HPO_4 3.4 g, KH_2PO_4 1.3 g, $MgCl_2{\cdot}6H_2O$ 0.2 g, $CaCl_2$ 0.1 g, NaCl 0.1 g, L-cysteine·HCl·H_2O 0.5 g, trace element 1 ml, Vitamin 1 ml. The pH of the medium should be at 7.0. Trace element was same with PFBM.

2.2. H_2 production from glucose by the mixed culture

The hydrogen production experiment was carried out in triplicate with 50 ml of the medium in 100 ml serum bottles, which were sealed by rubber plugs and filled with argon to maintain anaerobic conditions. The OD_{660} came to 2.49 after pre-culture of *R. faecalis* RLD-53 for 24 h, and The OD_{660} came to 2.68 after pre-culture of *C. butyricum* for 24 h. *C. butyricum* and RLD-53 were mixed in ratio of 1:100, 1:200, 1:300, 1:400, 1:500 and 1:600, respectively. Inoculants (5 ml) of mixed culture were put into sterile fresh mixed-culture medium. The bottles were shaken at 120 rpm/min at constant temperature of 35 °C; the light intensity on the outside surface of the bottles was maintained at 4000 lx by incandescent lamps (60 W).

The hydrogen production medium for mixed culture consists of per l.0 L, glucose 9.0 g, sodium glutamate 1.0 g, yeast extract 1 g, K_2HPO_4 3.4 g, KH_2PO_4 1.3 g, $MgCl_2{\cdot}6H_2O$ 0.2 g, $CaCl_2$ 0.1 g, $FeSO_4{\cdot}7H_2O$ 0.012 g, NaCl 0.1 g, EDTA–Na 0.1 g, L-cysteine.HCl·H_2O 0.5 g, trace element 1 ml, Vitamin 1 ml. The pH of the medium should be at 7.0. Trace element was same with PFBM.

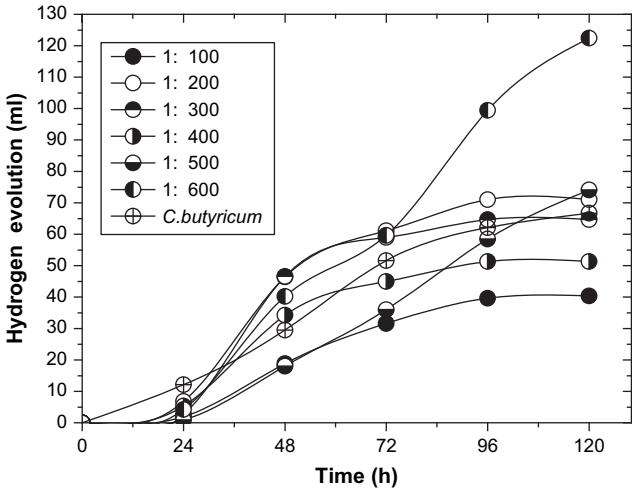


Fig. 1 – Hydrogen production by mixed culture of *C. butyricum* and RLD-53 in different ratio.

2.3. Analytical methods

Concentrations of glucose in the supernatants of culture broth were determined by the oxidase method. The volatile fatty acids in supernatant of the culture broth, and H_2 analysis in evolved gas were determined according to the method of Xing et al. [9]. The light intensity was measured by using a digital luxmeter (TES1330A, Junkai Co.). Cell concentration was determined by an Amersham pharmacia biotech ultrospec 34300 UV/Vis spectrophotometer.

3. Results and discussion

3.1. Hydrogen production by mixed culture

Fig. 1 showed that hydrogen yield increased with the ratio of dark-photo bacteria from 1:100 to 1:200. However, it decreased

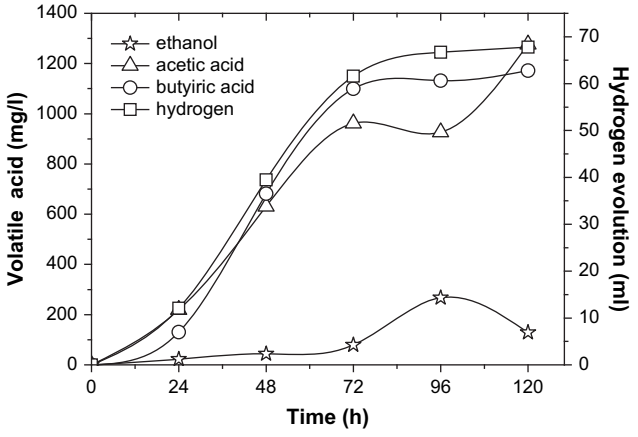


Fig. 2 – VFAs production by pure culture *C. butyricum*.

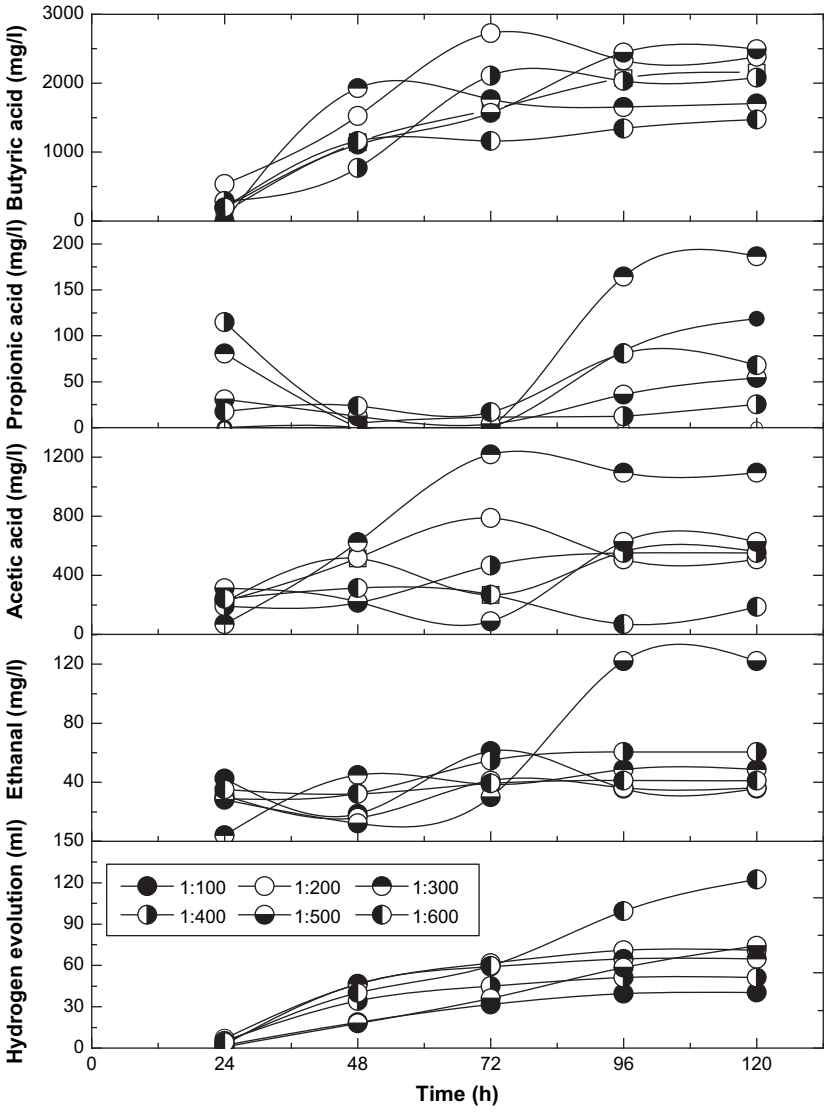


Fig. 3 – VFAs and H_2 production in different ratios of *C. butyricum* to RLD-53.

with the ratio from 1:300 to 1:400. The hydrogen production of 1:200 and 1:500 was on the same level, hydrogen yield was about 70 ml (1.4 ml-H_2/ml-culture) and it was also close to the pure culture of *C. butyricum* and RLD-53. The maximum cumulative hydrogen volume of 122.4 ml (2.448 ml-H_2/ml-culture) was obtained when the ratio of *C. butyricum* and RLD-53 was 1:600. The co-culture having the *R. sphaeroides*:*C. butyricum* ratio of 5.9:1 (cell number ratios) had the highest hydrogen yield (0.60 ml/ml medium) [6]. The hydrogen production was similar in mixed culture with different ratio within 24 h, this indicated that lag phase of hydrogen production almost was same for all tests. Between 48 h, lower hydrogen production yield was observed when in the ratio of 1:100 and 1:500, compared with that of pure culture *C. butyricum*. This can be explained by *C. butyricum* as the main hydrogen producer in mixed-culture system. After 48 h, hydrogen yield of 1:200, 1:300, 1:400 and 1:600 was higher than that of pure culture *C. butyricum*, this seemed that the dark-photo bacteria cooperated for hydrogen production and the increase of amount of photo-fermentation bacteria was favorable to mixed-culture hydrogen production. Subsequently, the amount of the volatile fatty acids from *C. butyricum* increased gradually, although volatile fatty acids were utilized by photo-fermentation bacteria, but the utilization rate of VFAs by photo-fermentation bacteria was lower than the rate of acidification by *C. butyricum* led to pH fell to low level and this greatly inhibited the growth of photo-fermentation bacteria RLD-53 and its consumption of VFAs, resulting in the decrease of hydrogen production by these two bacteria and at 120 h hydrogen production was stopped. The highest hydrogen production yield of 1.98 mol H_2/mol glucose was obtained in the mixing ratio of 1:600, and the rate was up to 0.5 ml/ml culture/day.

3.2. *Hydrogen production by pure culture of* C. butyricum

Fig. 2 showed an increasing trend between hydrogen production and VFAs production in pure culture *C. butyricum*. Moreover, hydrogen and VFAs production by *C. butyricum* in

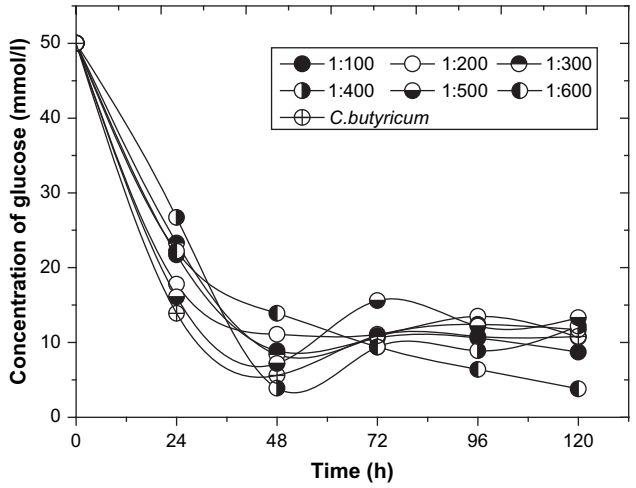


Fig. 4 – Utilization of glucose during hydrogen production in different ratio of *C. butyricum* to RLD-53.

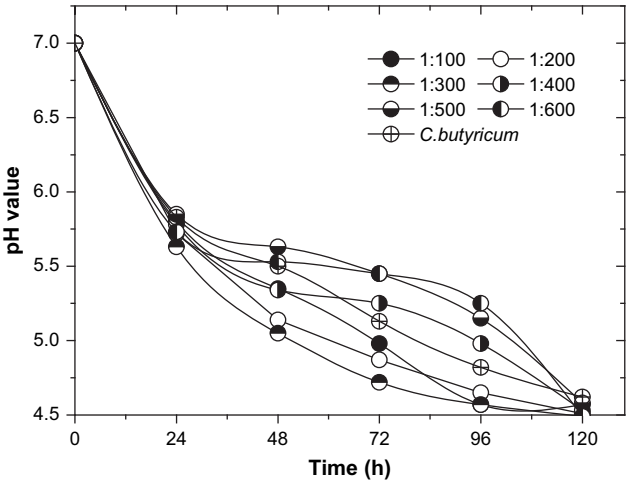


Fig. 5 – The change of fermentative pH during hydrogen production in different ratio of *C. butyricum* to RLD-53.

lag phase began at about 12 h. Hydrogen yield was 1.8 mol H_2/mol glucose. However, lag phase of photo-fermentation bacteria for hydrogen production was about 24 h and growth rate of dark-bacteria was faster than that of photo-bacteria. So, in order to keep a balance for cooperation in their amount, RLD-53 needed a great quantity addition in culture system. As a result, pH fell with the accumulation of VFAs and activity of bacteria were inhibited. That led to the reduction of hydrogen and the increase of VFAs at one time and until hydrogen production stopped. Hydrogen yield of mixed culture corresponding to the hydrogen yield of pure culture of *C. butyricum* and it was slightly higher than that of pure culture *C. butyricum*.

3.3. *Volatile acids production by mixed culture*

It was concluded that the similar results appeared between the mixed culture in ratio of 1:100 (*C. butyricum* to RLD-53) and pure culture of *C. butyricum* in Fig. 3. This observation turned out those photo-fermentative bacteria in the mixed culture with ratio of 1:100 used acetic acid and butyric acid as substrates. However, pH was decreasing because of the faster production rate of butyric acid than acetic acid and this was bad for the cooperation in hydrogen production. Eventually hydrogen production stopped at 120 h. With the increase of the amount of photo-fermentation bacteria in the mixed system, after 48 h produced acetic and butyric acids in the ratio of 1:600 did not increase and they were lower than pure culture of *C. butyricum*, and pH decreased slowly. This indicated that photo-fermentation bacteria utilized produced acetic and butyric acids and played main part in this period. As a result, acetic acid and butyric acid were consumed and their cooperation was formed in a way like food chain. With the increase of amount of photo-fermentation bacteria, produced hydrogen yield increased gradually. The pH fell to about 4.5 when glucose was consumed excessively. It was suggested that the utilization rate of acids by photo-fermentative bacteria was lower than production rate of acids by *C. butyricum*. The reason for pH falling was the higher production rate of VFAs compared to utilization rate. So, the cell growth of RLD-53 was restrained and hydrogen production stopped when pH in lower level, and the substrates was not fully consumed.

3.4. *The utilization of glucose and change of pH during mixed culture*

The utilization of glucose during mixed-culture hydrogen production is shown in Fig. 4. In the beginning 24 h glucose was consumed to half but generated hydrogen was a little. It was suggested dark-fermentative bacteria were using glucose for growth and hydrogen production. At 48 h, glucose was still decreasing, and all tests generated hydrogen. From 72 h glucose presented an increasing trend and VFAs were consumed at the same time. It was implied photo-fermentative bacteria were using volatile acids.

The changes of pH are shown in Fig. 5. The end pH value maintained at about 4.5 in all tests. It was concluded that the increase of the amount of RLD-53 not avoided the acidification of the mixed-culture system, because the production rate of volatile acids was faster than utilization rate of volatile acids by RLD-53, pH drop sharply in lower level and the activity of photo-fermentative bacteria was limited, and produced VFAs by *C. butyricum* were not fully used. So there were still problems in the direct mixed-culture pattern of photo- and dark-fermentative bacteria. The pH plays a major role in biological H_2 production [10]. It needs further research and efforts would be necessary to improve pH value of the fermentation system during mixed-culture process.

4. Conclusions

During hydrogen production in batch mixed culture, glucose was utilized firstly by *C. butyricum* and volatile acids were produced, RLD-53 began to utilize acetic acid and butyric acid for growth and hydrogen production. It showed the change of

substrates in food chain. But the production rate of volatile acids was faster than utilization rate by RLD-53. As a result pH fell quickly in the system and RLD-53 suffered from the toxic effect, microbial growths were limited and hydrogen production stopped. The optimal ratio was 1:600 (*C. butyricum* to RLD-53) for mixed-culture hydrogen production. The maximum hydrogen yield of 122.4 ml/vessel was obtained and hydrogen production rate was 0.5 ml H_2/ml culture/day.

Acknowledgements

This work was supported by the National Natural Science Fund of China (No. 30470054) and National Natural Science Key Fund of China (No. 50638020). The authors would like to thank the National High Technology Research and Development Program of China (863 Program, Grant No. 2006AA05Z109) and China Postdoctoral Science Foundation funded project (Grant No. 20070410910) for their support for this study. Results could be of great significance for further pilot studies of mixed culture.

REFERENCES

[1] Weetall H, Sharma BP, Detar CC. Photometabolic production of H_2 from organic substrates by free and immobilized mixed cultures of *Rhodospirillum rubrum* and *Klebsiella pneumoniae*. Biotechnol Bioeng 1981;23:605–11.

[2] Odom JM, Wall JD. Photoproduction of H_2 by an anaerobic bacterial co-culture. Appl Environ Microbiol 1983;45:1300–5.

[3] Miyake J, Mao XY, Kawamura S. Hydrogen photoproduction from glucose by a co-culture of a photosynthetic bacteria and *Clostridium butyricum*. J Ferment Technol 1984;62(6):531–5.

[4] Yokoi H, Mori S, Hirose J, Hayashi S, Takasoki Y. H_2 production from starch by a mixed culture of *Clostridium butyricum* and *Rhodobacter* sp. M-19. Biotechnol Lett 1998;20: 895–9.

[5] Asada Y, Tokumoto M, Aihara Y. Hydrogen production by co-cultures of *Lactobacillus* and a photosynthetic bacterium, *Rhodobacter sphaeroides* RV. Int J Hydrogen Energy 2006;31: 1509–13.

[6] Fang HHP, Zh Heguang u, Zhang Tong. Phototrophic hydrogen production from glucose by pure and co-cultures of *Clostridium butyricum* and *Rhodobacter sphaeroides*. Int J Hydrogen Energy 2006;31:2223–30.

[7] Ren NQ, Liu BF, Ding J, Xie GJ. Hydrogen production with *R. faecalis* RLD-53 isolated from freshwater pond sludge. Bioresource Technology 2009;100:484–7.

[8] Zhang DM, Yang HF, Huang ZY, Zhang W, Liu SJ. *Rhodopseudomonas faecalis* sp. nov., a phototrophic bacterium isolated from an anaerobic reactor that digests chicken faeces. Int J Syst Evol Microbiol 2002;52:2055–60.

[9] Xing DF, Ren NQ, Wang AJ, Q L, Feng YJ, Ma F. Continuous hydrogen production of auto-aggregative *Ethanoligenens harbinense* YUAN-3 under non-sterile condition. Int J Hydrogen Energy 2008;33:1489–95.

[10] Venkata Mohan S, Srikanth S, Dinakar P, Sarma PN. Photobiological hydrogen production by the adopted mixed culture: data enveloping analysis. Int J Hydrogen Energy 2007;. doi:10.1016/j.ijhydene.2007.10.033.