

Biohydrogen production by fermentive bacterium *Clostridium* sp. Tr2 using batch fermenter system controlled pH under dark fermentation

Sản xuất hydro sinh học nhờ vi khuẩn lên men Clostridium sp. Tr2 bằng cách lên men tối theo mẻ có kiểm soát pH

Research article

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Limitation of fuels reserves and contribution of fossil fuels to the greenhouse effect leads to develop a new, clean and sustainable energy. Among the various options, biohydrogen appears as a promising alternative energy source. The fermentative hydrogen production process holds a great promise for commercial processes. Hydrogen production by fermentative bacteria is a very complex and greatly influenced by pH. This paper presents biohydrogen production by bacterial strain *Clostridium* sp. Tr2. Operational pH strongly affected its hydrogen production. Its gas production rate as well as obtained gas product were roughly increase twice under controlled pH at 6 than non-controlled condition. Dark fermentation for hydrogen production of strain Tr2 was performed under bottle as well as automatic fermenter scale under optimal nutritional and environmental conditions at 30°C, initial pH at 6.5, then pH was controlled at 6 for bioreactor scale (BioFlo 110). Bioreactor scale was much better for hydrogen production of strain Tr2. *Clostridium* sp. Tr2 produced 0.74 L hydro (L medium)⁻¹ occupying 72.6 % of total gas under bottle scale while it produced 2.94 L hydro (L medium)⁻¹ occupying 95.82 % of total gas under fermenter scale. Its maximum obtained hydrogen yield of *Clostridium* sp. Tr2 under bioreactor scale Bioflo 110 in optimal medium with controlled pH 6 was 2.31 mol hydro (mol glucose)⁻¹.

Dự trữ nhiên liệu có giới hạn và việc sử dụng nhiên liệu hoá thạch góp phần không nhỏ gây hiệu ứng nhà kính dẫn đến cần phải phát triển năng lượng mới, sạch và bền vững. Trong số các giải pháp, hydro sinh học xuất hiện như một nguồn năng lượng thay thế đầy hứa hẹn. Quá trình lên men sản xuất hydro có tiềm năng lớn để áp dụng trong sản xuất thương mại. Tuy nhiên quá trình này rất phức tạp và chịu ảnh hưởng lớn bởi pH. Nghiên cứu này trình bày sản xuất hydro sinh học do chủng vi khuẩn *Clostridium* sp. Tr2. Quá trình sản xuất hydro của chủng này bị ảnh hưởng mạnh mẽ bởi pH thay đổi trong quá trình lên men. Tốc độ tạo khí cũng như lượng khí thu được của chủng này tăng gần gấp đôi trong môi trường có duy trì pH ở pH 6 so với môi trường không kiểm soát pH. Quá trình lên men tối sản xuất hydro của chủng Tr2 được thực hiện ở quy mô bình thí nghiệm cũng như bình lên men tự động trong điều kiện môi trường tối ưu ở 30°C, pH ban đầu 6.5, ở quy mô bình lên men tự động (BioFlo 110), pH môi trường sau đó được duy trì ổn định ở pH 6. Lên men sản xuất hydro của chủng Tr2 trong bình lên men tự động tốt hơn rất nhiều so với lên men trong bình thí nghiệm. *Clostridium* sp. Tr2 chỉ tạo ra được 0,74 L hydro (L medium)⁻¹ chiếm 72,6 % tổng thể tích khí thu được ở điều kiện lên men bình thí nghiệm trong khi chủng này sản xuất được 2,94 L hydro (L medium)⁻¹ chiếm 95,82 % tổng thể tích khí ở điều kiện lên men tự động. Sản lượng hydro thu được lớn nhất của chủng này trong bình lên men tự động BioFlo 110 trong môi trường tối ưu có kiểm soát pH tại pH 6 là 2,31 mol hydro (mol glucose)⁻¹.

Keywords: biohydrogen, dark fermentation, batch condition, *Clostridium*, Vietnam

1. Introduction

Environmental pollution due to the use of fossil fuels as well as their short fall have led most of the countries to invest in research on alternative sources of energy to resolve the issues of environment protection and energy security, and to slow down the climate change. Amongst the different alternatives, biohydrogen appears as a promising energy source that is cost-effective, environment-friendly and renewable. It has highest specific energy content per unit mass of any known fuel and produces only water as the by-product, when it is combusted as a fuel or converted to electricity (Kotay and Das, 2008; Mudhoo et al., 2011; Kothari et al., 2012; Gupta et al., 2013; Shah et al., 2017). Biohydrogen production can be achieved by fermentative hydrogen production (dark fermentation) as well as photosynthetic hydrogen production (photo-fermentation). However, a fermentative hydrogen production process by strict/facultative anaerobes holds a great promise for commercial processes. There are a number of advantages of this process such as using the vast majority of substrates, no inhibitory effect of oxygen, higher hydrogen production efficiency, higher hydrogen production stability, higher feasibility for industrialization, simpler control requirement, and lower operating costs (Das and Veziroglu, 2008; Kotay and Das, 2008; Lee et al., 2010; Ntaikou et al., 2010; Ren et al., 2011; Show et al., 2012; De Gioannis et al., 2013; Wong et al., 2014). Thus, it has received considerable attention during the recent years.

Fermentative hydrogen production is a very complex process and is greatly influenced by many nutrient and environmental factors, especially pH. Various researchers have shown the influence of pH to improve hydrogen yields and rate of productivity (Skonieczny and Yargeau, 2009; Khanal et al., 2011; Liu et al., 2011; Chu et al., 2013). In the present study, the effect of operational pH on hydrogen production was investigated and the dark fermentation for hydrogen production under different scales (bottle and automatic fermentor) was performed using the newly hydrogen-producing facultative anaerobe (designated as *Clostridium* sp. Tr2) from dung-feces in Vietnam.

2. Materials and methods

2.1. Strain and medium

The mesophilic, facultative strain Tr2 used in this study was identified as *Clostridium* sp. Tr2 based on 16S rRNA analysis (Nguyen et al., 2013). This strain was taken from the culture collection of IBT (Institute of Biotechnology), VAST (Vietnam Academy of Science and Technology).

The basic medium used in the present work was NMV medium adjusted basing on results of previous works (Nguyen et al., 2012; Dang et al., 2013).

2.2. Cultivation

Fermentation under the optimal condition: The optimal condition for strain Tr2 dark fermentation was defined in our previous reports (Nguyen et al., 2012; Dang et al., 2013). Then dark fermentation of strain Tr2 was performed in 600ml serum bottles that contained 500ml optimal NMV medium with 10% overnight culture that was in log phase of growth under 30°C, initial pH 6.5. These bottles were air-sealed with butyl rubber stopper and tied with aluminium seal cap. The optimal medium for hydrogen producing bacterium *Clostridium* sp. Tr2 is NMV medium basing on results Nguyen et al., 2012; Dang et al., 2013 in which some critical adjustments are glucose 10.18 g (L)⁻¹, yeast extract 2.5 g (L)⁻¹, no need meat extract, FeSO₄ .7H₂O 58 mg (L)⁻¹. During dark fermentation at 600 ml bottle scale, the cell density and the volume of gas mixture were examined at each 4 h interval until hydrogen production was ceased. The quality of gas product was analysed at the end of experiment.

For controlled pH experiment: Both experiments (non-controlled and controlled pH) were carried out in 120 ml serum bottles with 100 ml medium with initial pH 6.5. These bottles were air-sealed with butyl rubber stopper and tied with aluminium seal cap. Experiments were performed in optimal NMV media (mentioned above) for strain Tr.2 with 10% (v/v) inoculum of pre-culture that was in log phase of growth under facultative anaerobic condition at 30°C, initial pH 6.5. pH media during fermentative process were estimated 4 h per time. When the strain grew, pH would be decreased. When pH reduced at pH 6.0, one bottle was keeping at constant value pH 6.0 by feeding NaOH (5M), the other was not controlled pH until hydrogen production was ceased. At each 4 h interval, the cell density and the total gas volume were also measured.

For batch 7 L automatic fermentor system scale (BioFlo 110): Strain Tr2 was pre-cultured twice, first in 120 ml serum bottles with 100 ml medium and then second in 600 ml serum bottles with 500 ml medium under facultative anaerobic fermentation at 30°C. The hydrogen production capacity was checked during pre-cultivation. Ten percent (v/v) of second pre-culture was used as inoculum for dark fermentation in 7 L automatic fermentor containing 5.6 L medium under facultative condition. The temperature was maintained at 30°C by a heating jacket. pH was adjusted to 6.5 at the beginning of experiment. When pH was down to 6.0, it was controlled at 6.0 through peristaltic pumps connected with alkali reservoirs (5M NaOH) until hydrogen production ceased. The evolved gas mixture was measured 2 h per time. For analysis, 10 mL of the fermentation broth was drawn at each 2 h interval. The mixed liquor samples were used to analyse the glucose consumption and to measure the cell density. The volume of gas product was continuously collected and recorded at each 2 h interval. The quality of gas product and by-products volatile fatty acids, ethanol and butanol were analysed at the end of experiment. The dark fermentation was carried out until hydrogen production was ceased.

2.3. Analyses

Bacteria growth; glucose concentration; volume of gas mixture were performed as mentioned in Nguyen et al., 2014.

The quantity and composition of gas products (mainly, H_2 and a little of CO_2 and H_2S) were determined in a gas chromatograph GC-TCD (Thermo Trace GC-Thermo Electro-USA) equipped with a thermal conductivity detector (TCD) and a column packed Molecularsieve 13X 5m. The operational temperatures of the oven and the detector were $50^\circ C$ and $200^\circ C$, respectively. Helium was used as the carrier gas at a flow rate of 25 mLmin^{-1} .

The concentrations of volatile fatty acids (acetic acid and butyric acid) as by-products were analyzed by high pressure liquid chromatography HPLC-PDA (Shimadzu, Japan) equipped with UV detector at 210 nm and column Supecogel C610H coated with 1% H_3PO_4 at a flow rate of 0.5 mLmin^{-1} . The temperature of the injection port was $30^\circ C$.

The by-products ethanol and butanol were also detected by GC-MS (Polaris Q Thermo Electro-USA) with a col-

umn packed HP1-MS-(30m x 0.15mm x 0.25 μm). Helium was used as the carrier gas at a flow rate of 1 mLmin^{-1} . The temperature of the injection and interface oven was $200^\circ C$. The temperature for column oven: starting temperature $40^\circ C$, temperature was raised up $6^\circ C$ per min, then kept constantly at $230^\circ C$ for 10 min.

3. Results and discussion

3.1. Biohydrogen production of strain Tr2 by dark fermentation under bottle scale

The results of the dark fermentation in optimal NMV medium of strain Tr.2 was shown in Figure 1&2. These results emphasized that H_2 production of strain Tr2 was also accompanied with growth and glucose consumption. H_2 production began when strain Tr2 entered the early exponential growth phase and rate of H_2 production reached a maximum in the late exponential growth phase. These findings are similar with our previous report of strain Trau DAT (Nguyen et al., 2014).

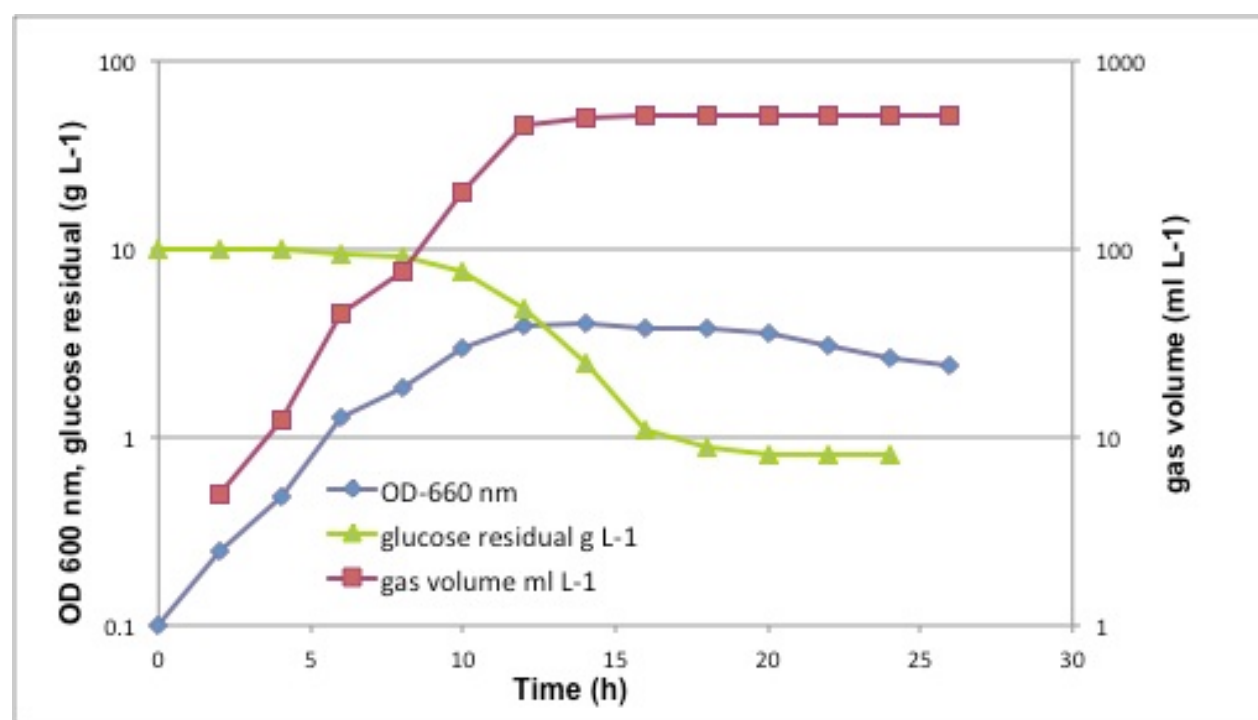


Figure 1. Capability of growth, hydrogen production, glucose consumption of strain Tr2 under optimal condition dark fermentation at bottle scale

Figure 1 also pointed out that strain Tr2 consumed about $9.3 \text{ g glucose (L)}^{-1}$ (initial glucose concentration was 10.18 g (L)^{-1}) to produce $1020 \text{ ml gas mixture (L medium)}^{-1}$ (Figure 1). The gas analyses showed that the gas mixture contains hydrogen, CO_2 and H_2S with 72.6%, 1.2% and 26.1%, respectively (Figure 2). Based on these results, it was realized that real hydrogen volume was produced by strain Tr.2 under bottle scale on optimal NMV medium was $740 \text{ ml (L medium)}^{-1}$.

In comparison with our previous reported strain Trau DAT (Nguyen et al., 2014), the H_2 obtained volume of strain Tr2 is higher than strain Trau DAT even though the gas quality is lower. However, the difference wasn't so much distinguished. It implies that both our thermophilic, anaerobic bacterium *Thermoanaerobacterium aciditolerans* Trau DAT and mesophilic, facultative *Clostridium* sp. Tr2 are effective hydrogen producers using glucose as carbon source (96.67% and 91.36% glucose consumption for each respective strain).

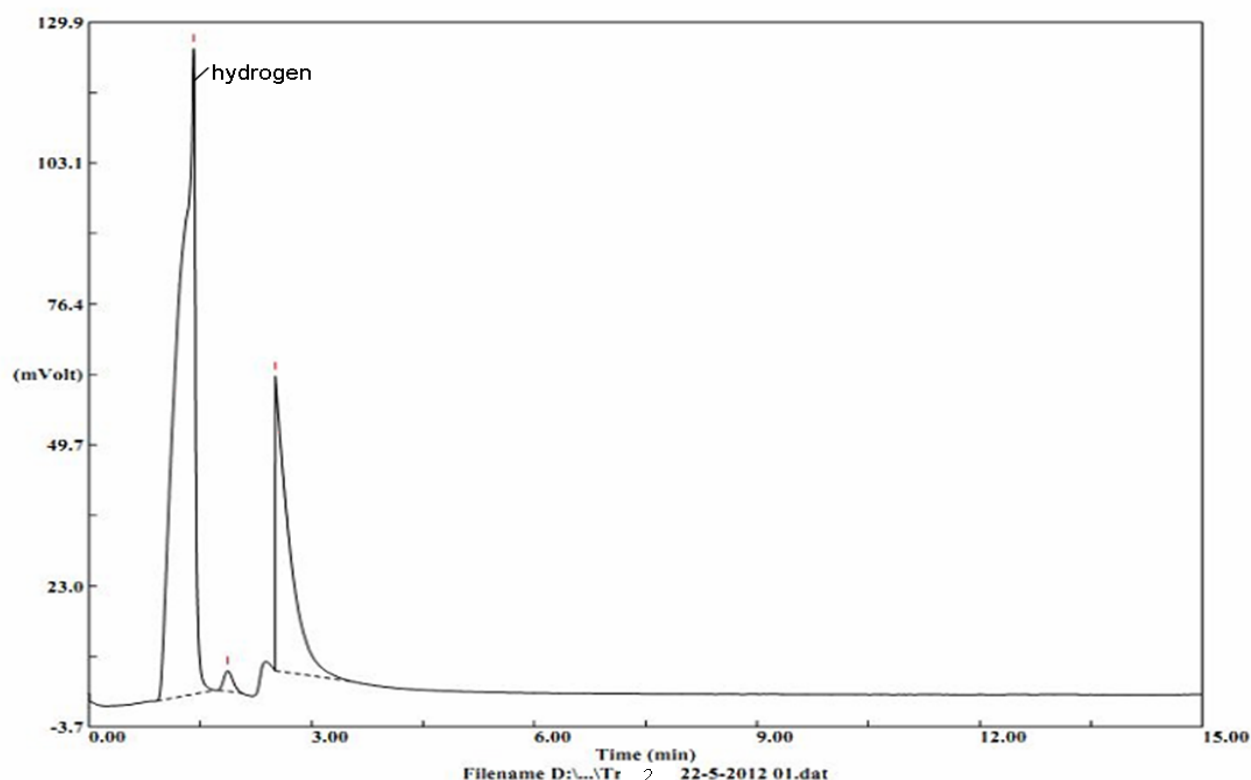


Figure 2. Chromatograph of gas product of strain Tr2 under optimal condition dark fermentation at bottle scale

3.2. Hydrogen production of strain Tr2 under controlled pH condition

Many papers including our previous paper indicated that dark fermentation by hydrogen producing bacteria depends on not only nutritional factors but also pH [Davila-Vazquez et al., 2008; Li et al., 2010; Khanna et al., 2011; Nguyen et al., 2012; 2014; Dang et al., 2013]. In our early study, it was found that pH of the medium was descended

during the fermentation process of strain Tr2. It was also found that the maximum hydrogen production was observed when pH was 6.0. At pH 3-4, the hydrogen production of strain Tr2 was ceased (data not shown). Thus, in this study, the experiment in which initial pH 6.5 was set up and then operational pH was controlled at 6.0 was performed in order to find out whether controlled pH could increase the hydrogen production potential of strain Tr2.

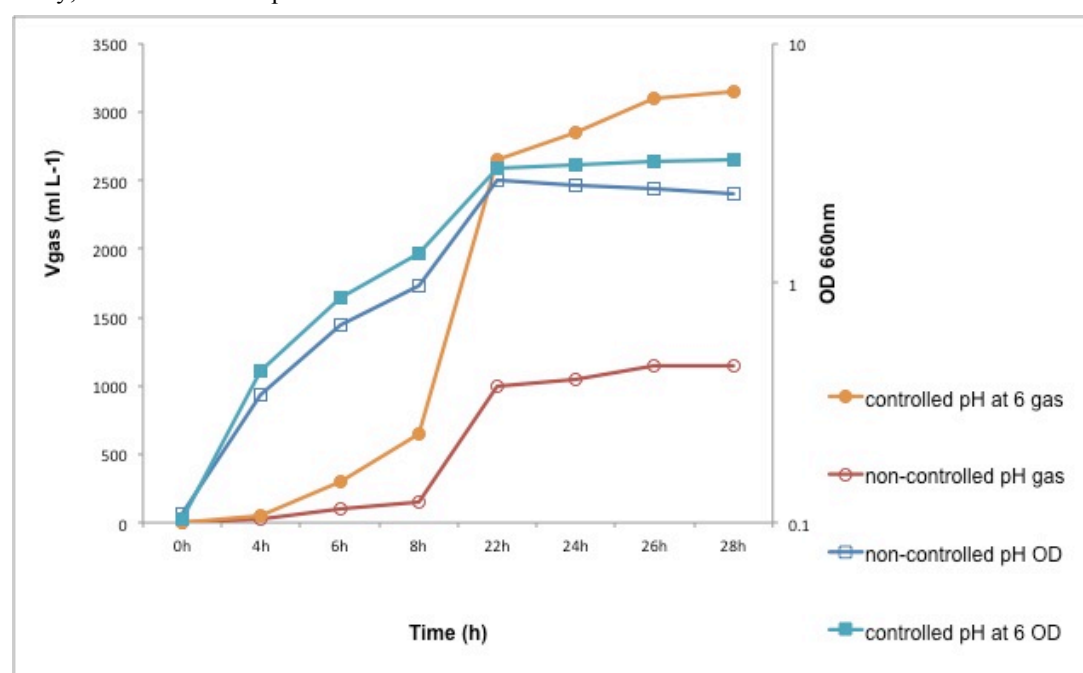


Figure 3. Effect of pH control on the growth and hydrogen production of strain Tr2

The result shown in Figure 3 indicated that hydrogen production capacity of strain Tr2 was highly affected by operational pH. The gas production rate ($\text{ml h}^{-1} \text{ L medium}^{-1}$) of strain Tr2 was 71.4 and 41 under controlled and non-controlled pH condition, respectively. The gas volume produced by strain Tr2 under controlled pH was much higher than that under non-controlled pH. It implied that hydrogen fermentation condition was favorably maintained by pH control in the cultures. These findings are in agreement with results of Lee et al. (2008), Alalavah et al. (2009) that hydrogen yield is maximum if dark fermentation of hydrogen producing bacteria is started with initial pH at 6.5 and keep the constant at pH 6. Our results also were good evidences for the conclusion that constant pH improve hydrogen yields and rate of productivity

(Skonieczny and Yargeau, 2009; Li et al., 2010; Khanna et al., 2011; Nguyen et al., 2014).

3.3. Dark fermentation for hydrogen production at fermenter scale

Basing on above result and our previous reports [Nguyen et al., 2012; Dang et al., 2014], the dark fermentation of strain Tr2 was carried out at automatic fermenter system scale (Bio-Flo 110) using optimal NMV medium with some critical adjustments: 10.18 g (L)^{-1} glucose, 2.5 g (L)^{-1} yeast extract, $58 \text{ mg (L)}^{-1} \text{ FeSO}_4 \cdot 7\text{H}_2\text{O}$, meat extract 0 g (L)^{-1} , NaCl 0 g (L)^{-1} , 10% log-phase overnight second inoculums (v/v), initial pH 6.5 and then pH was automatically controlled at 6.0 during fermentative process.

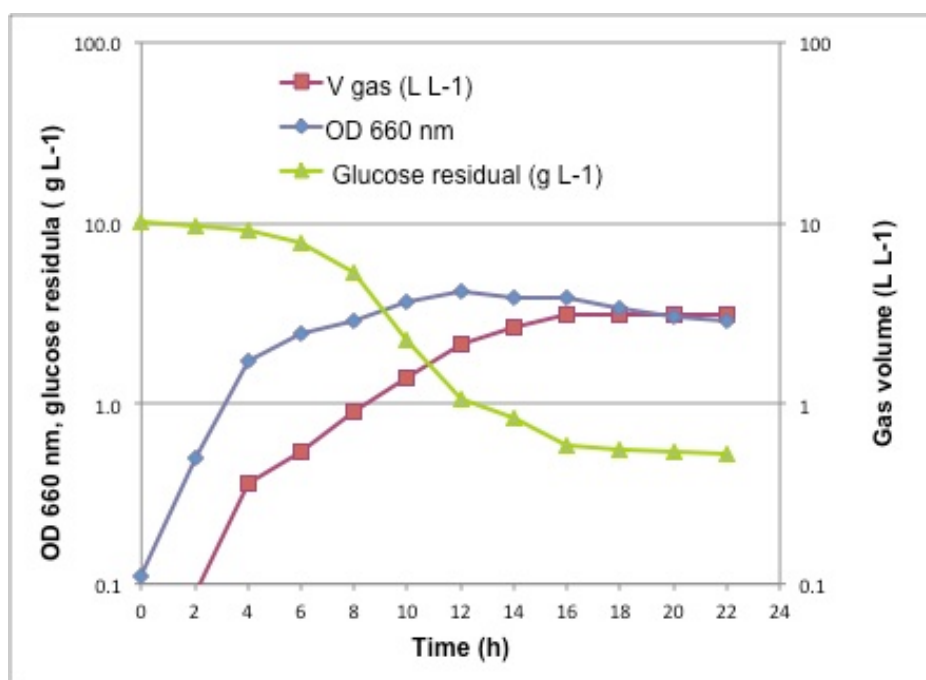


Figure 4. Capability of growth, hydrogen production, glucose consumption of strain Tr2 under optimal condition dark fermentation in automatic fermenter system

The results represented in Figure 4 show that strain Tr2 nearly had no lag phase and went intermediately to log phase. Then, it grew quickly and entered the stationary phase after cultivating 12 hours. Along with the growth, the gas product was generated after 2 hours cultivation, then was quickly enhanced since 6 hours fermentation. The total gas volume reached when experiment was finished was $3.07 \text{ L (L medium)}^{-1}$.

These obtained results emphasized that strain Tr2 grew faster under fermenter scale than under bottle one. It resulted from twice pre-cultivation and automatic controlled during main fermentation in Bioflo fermenter. Twice pre-cultivation resulted in that cells were more homogeneous and almost were in the log phase so there is nearly no need time for the lag phase when cells were cultivated in main fermentation experiment. In addition, the amount of gas produced by strain Tr2 in this condition was more than bottle one. This result is obtained by maintaining the operating pH at pH 6. It can be said that twice pre-

cultivation and maintaining pH 6 help shorten the fermentation time and increase the amount of obtained gas, then lead to increase yield of fermentation.

Analysis of the by-products of fermentation showed that the by-products included acid acetic, acid butyric and butanol with little amount. Analyses of gas products showed that hydrogen mainly occupied (95.82%) in comparison with CO_2 (2.14%) and H_2S (2.03%) (Figure 5). These implied that strain Tr2 mainly fermented to produce hydrogen. Analyses of gas products also showed that the hydrogen quality obtained from these strains (95.82%) under fermenter scale was significantly higher than under bottle scale (72.6%). And the gas volume collected from fermenter ($3.07 \text{ L (L medium)}^{-1}$) was also much higher than from bottle ($0.74 \text{ L (L medium)}^{-1}$). These findings pointed out that the bigger scale, the more automatic control, the more controlled pH resulted the more hydrogen quality and yield in agreement with Nguyen et al., 2014.

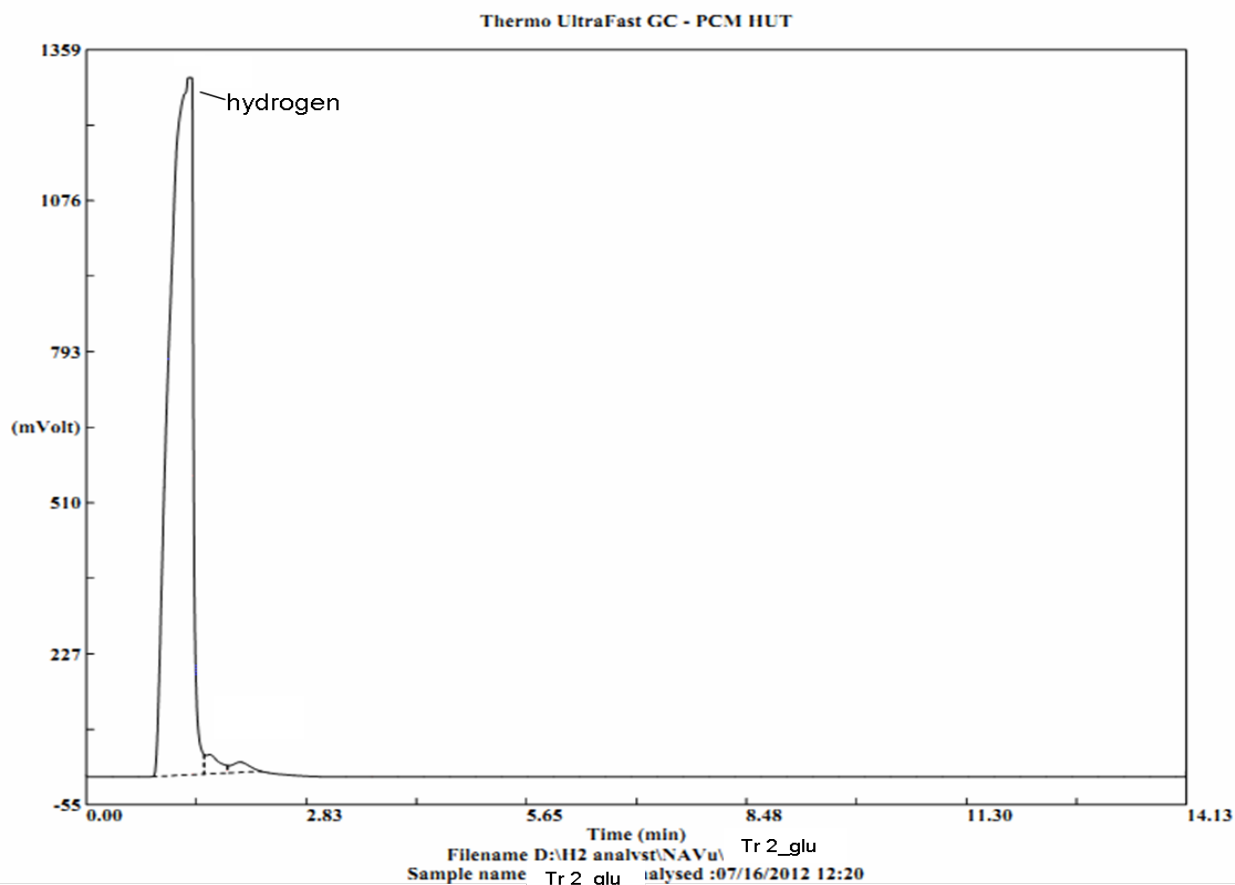


Figure 5. Chromatograph of gas product of strain Tr2 under optimal condition dark fermentation at fermentor scale

Results (Figure 4&5) also showed that strain Tr2 consumed 9.6 g (L)^{-1} glucose (94.3%) to produce $3.07 \text{ L gas (L)}^{-1}$ in which H_2 occupied 95.82%. Thus, hydrogen yield of strain Tr2 was $2.23 \text{ mol hydro (mol glucose)}^{-1}$. Compared to the theory that 1 mol of glucose can produce 2-4 mol of hydrogen by dark fermentation, the yield of hydrogen fermentation of the studied strains is relatively high in comparison with previous reports [Alalayah et al., 2009; Oh et al., 2009; Khanna et al., 2011; Liu et al., Nguyen et al., 2014].

4. Conclusion

Obtained results of the present study showed that the hydrogen production capacity of *Clostridium* sp. Tr2 highly depended on the fermentation condition, especially pH. Fermentation started with initial pH at 6.5 and kept the constant at pH 6 enhanced the hydrogen production yield and rate of strain Tr2. In addition, twice increasing volume scale pre-cultivation, automatic control fermentor could also contribute to increase the quality and volume of obtained hydrogen product. At bottle scale, strain Tr2 produced $740 \text{ ml H}_2 \text{ (L medium)}^{-1}$ occupied 72.6% of total gas. The maximum volume of total gas produced by the strain Tr2 was $3.07 \text{ L (L medium)}^{-1}$ corresponding to $2.94 \text{ L H}_2 \text{ (L medium)}^{-1}$, equivalent to $2.23 \text{ mol H}_2 \text{ (mol glucose)}^{-1}$ under the optimal condition and maintainable pH 6.0 in automatic fermentor system BioFlo 110.

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