Shahid Bahonar University of Kerman

Biomechanism and Bioenergy Research

Online ISSN: 2821-1855
Homepage: https://bbr.uk.ac.ir

Iranian Society of Agricultural Machinery Engineering and Mechanization

Regulation of Metabolic Pathway for Bio-Hydrogen Production in Dark Fermentation via Redox Potential

Saeed Khesareh[1], **Seyed-Ahmad Ataei**[1]✉

[1] Chemical Engineering Department, Faculty of Engineering, Shahid Bahonar University of Kerman, Iran.
✉ Corresponding author: ataei@uk.ac.ir

ARTICLE INFO

Article type:

Research Article

Article history:

Received 23 September2023

Received in revised form 09 November 2023

Accepted 18 December 2023

Available Online 28 December 2023

Keywords:

Biohydrogen; Dark fermentation; Oxidation potential; Metabolic pathway.

ABSTRACT

The ever increase in global population and consequently daily increase in energy consumption are casing various environmental pollution and worldwide climate changes. Replace fossil with different type of clean and renewable energy or decreasing the consumption of petroleum-based fuels will greatly reduce the hazardous effects of fossil fuels. Biohydrogen is a suitable alternative source of energy that can reduce dependency on conventional fuels. In this research the effect of the external oxido-reduction system on biohydrogen production from glucose fermentated in a dark medium was carried out and the effect of oxidation potential on biohydrogen production from clostridium acetobutylicum was investigated. The maximum hydrogen production rate and accumulative hydrogen were calculated using the modified Gompertz equation. Results show that the increase of voltage to 600 mV, leads to an increase of 25% in hydrogen production rate and a 19% increase in yield. It was also observed that the amount of undesired end products like ethanol and lactate decreased with the increase of oxidation potential and the acetate to butyrate ratio (A/B) increased from 0.82 to 1.52 when the voltage was raised to 600 mV.

Cite this article: Khesareh, S., Ataei, S. A. (2023). Regulation of Metabolic Pathway for Bio-Hydrogen Production in Dark Fermentation via Redox Potential. *Biomechanism and Bioenergy Research*, *2*(2), 10-18. https://doi.org/10.22103/BBR.2023.22231.1054

Publisher: Shahid Bahonar University of Kerman
DOI: https://doi.org/10.22103/BBR.2023.22231.1054

INTRODUCTION

Bio-hydrogen is a suitable candidate as a substitute for fossil fuels (Guo et al., 2010). Diminish of fossil fuels reserves, and emission of greenhouse gases and pollutants like CO_X, NO_X, SO_X, CxHy, and ash, have infused extensive studies to acquire alternative energy sources that do not cause pollution (Das & Veziroğlu, 2001). Hydrogen has a high conversion efficiency, negligible generation of pollutants on combustion, and high energy content by three times compared to fossil fuels. Thus the choice of hydrogen as a first candidate for future energy carriers seems to be rational (Hallenbeck, 2009; Bisaillon et al., 2006; Li et al., 2020). Unfortunately, steam reforming of natural gas and coal gasification are the main commercial hydrogen production methods (Elam et al., 2003) which are energy intensive and not environmentally friendly (Levin et al., 2007). Thus, these conventional methods have several drawbacks and a sustainable method has to be replaced (Konieczny et al., 2008; Liu et al., 2014). It seems that biological hydrogen production is the best alternative to conventional methods because these processes require a lower energy supply, and in addition to their clean nature of the process, they can be used for waste treatments and pollutant remediator (Arimi et al., 2015; D. H. Kim et al., 2011).

Significant attention has been received to the dark fermentation method due to its higher hydrogen production rate, lower operational cost, and simpler reactor technology compared to other methods (Antonopoulou et al., 2011; Dzulkarnain et al., 2022; Singh & Sarma, 2022).Organic compounds from the wide range of wastewater prepared for dark fermentation culture can be used as carbon sources for organic acids, CO_2, alcohols and H_2 generation (Ghimire et al., 2015). If the only end product of dark fermentation of hydrogen production is acetate, then The maximum theoretical yield is 4 moles H_2/ mole glucose (Balat, 2009; Koutra et al., 2020). Also, hydrogen quantity comes down when the production pathway of side-products such as ethanol, lactate and propionate are active because of the same substrate pyruvate (Cao et al., 2022). Thus, metabolic pathways regulation of hydrogen production by bacteria can increase the yield of hydrogen generation. Several approaches are employed to improve the hydrogen production yield through metabolic engineering. For example, the elimination of the reactions that compete for reductant, either reduced Nicotinamide adenine dinucleotide (NADH) or pyruvate, can increase the hydrogen production yield from the fermentation process (Hallenbeck & Ghosh, 2012). In one study, a 62% Improvement in H_2 production was reported through the redirection of metabolic pathways by blocking the formation of alcohol and some organic acids in Enterobacter cloacae IIT-BT 08. Another study used a genetic and metabolic approach for enhancing H_2 yield via the redirection of metabolic pathways of a C. butyricum strain.

The ethanol formation pathway was blocked by disruption of aad (encoding aldehyde-alcohol dehydrogenase) using a Clostron plasmid and H_2 yield increased by 20% through lactate and ethanol production pathway blocking (Cai et al., 2013). Elimination of the activity of any so-called uptake hydrogenases that could be present is another type of modification that might be efficient in increasing hydrogen production yields. For example, in one case, the elimination of hyd1 and hyd2 increases the H_2 production yield by 37% compared to the wild-type strain (Bisaillon et al., 2006). Chemical and genetic methods were applied in most of the research that reported metabolic pathway regulation in dark fermentation and there wasn't any report about the effect of the external oxido-reduction potential system. Thus, herein the effect of the external oxido-reduction system on biohydrogen production and other metabolites production was investigated.

MATERIAL AND METHODS

Microorganism

The necessary Clostridium acetobutylicum for the experiments was purchased from Persian Type Culture Collection, (PTCC 1456) in lyophilized form. Clostridium acetobutylicum species were cultivated in a pre-culture medium containing (g/l): glucose 6, peptone 10, yeast extract 0.6, $MgSO_4.7H_2O$ 0.25, K_2HPO_4 1, KH_2PO_4 1, and L-cysteine-HCl.H_2O 0.1. After anaerobic incubation at 37 °C for 48 hours, the prepared culture was used as inoculum.

Experimental setup

Batch-dark-fermentation experiments were carried out in three 500 mL Erlenmeyer with 250 mL medium, one of them used as a control sample and the others for anode and cathode half cells. A salt bridge containing KCl (2M) and agar was used to make an ion exchange between half cells. The connection between the- power supply and half cells was made by the platinum electrodes and a multimeter was used to adjust the-voltage. Oxidation reaction has occurred in the Erlenmeyer which is connected to the positive pole (anode) and a reduction reaction has occurred in Erlenmeyer connected to the negative pole (cathode). The conditions of all of the three Erlenmeyer's were the same as follows: the fermentation medium was prepared by (g/l): peptone 10, yeast extract 0.6, $MgSO_4.7H_2O$ 0.25, K_2HPO_4 1, KH_2PO_4 1, $FeSO_4.7H_2O$ 0.025, and L-cysteine-HCl.H_2O 0.1. The glucose concentration, temperature and initial pH of experiments were adjusted based on the optimization results of the previous work (Shaterzadeh & Ataei, 2017). pH was adjusted to 6.5 by adding HCl 2N and NaOH 1N. After sterilization, the medium was purged with nitrogen gas to remove oxygen in the headspace of the bottles and kept in an anaerobic condition. After the addition of inoculant (10%), the bottles were placed in an incubator at 35°C for 48 hrs. figure 1 shows this part briefly.

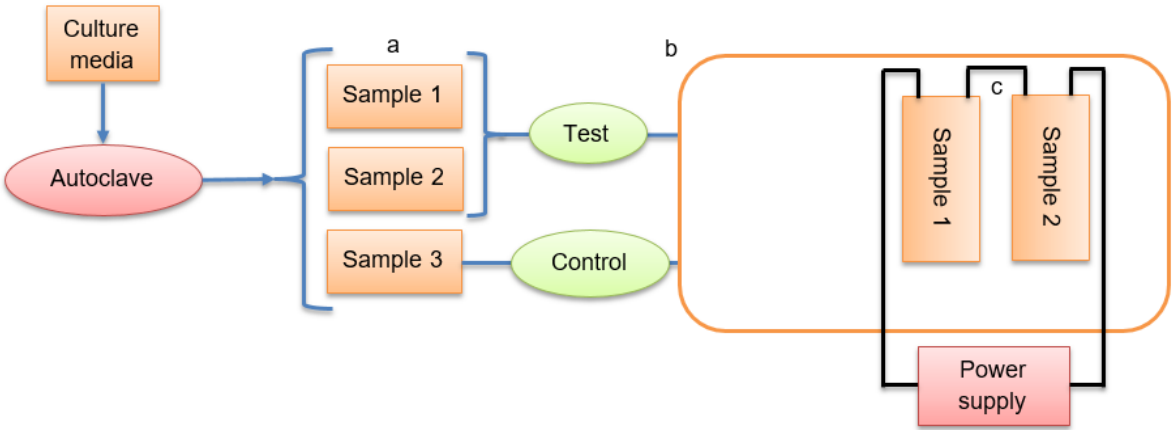

Figure 1. a) 10% of master culture (inoculant) added to each Erlenmeyer, b) incubation zone 35℃, c) salt bridge KCl 2M and Agar, d) cathode (reduction), e) anode (oxidation)

Analytical methods

The volume of the produced biogas is measured by the water displacement method. Hydrogen concentration is also measured by a gas chromatograph (Varian CM 3500) equipped with a thermal conductivity detector (TCD), stainless steel column (2 m × 3 mm) packed with molecular sieve 5 A, and using N_2 as the carrier gas. The operational temperatures of the column, injector and detector were kept at 50, 80 and 90 °C, respectively. The concentration of volatile fatty acids (VFAs) was measured in centrifuged (4000 rpm for 15 min) and filtered

samples (1.2 μm fiberglass filter) by a GC mass equipped with a 60m column (HP- 5CB) with an inner diameter of 0.25 mm.

Data analysis

The hydrogen production rate and yield are two important parameters to describe the hydrogen production process. The yield is mathematically defined as a ratio of moles of produced hydrogen to moles of consumed substrate or feed and the hydrogen production rate is the ratio of produced hydrogen volume (ml) on time unit (h) at reactor volume unit (l). The modified Gompertz equation (1) which was successfully applied to the cumulative hydrogen production in batch experiments (Jeong et al., 2008) is employed here to calculate the hydrogen production rate:

$$H(t) = P \times \exp\left[-\exp\left(Rm.\frac{e}{p}(t-\gamma)+1\right)\right] \qquad (1)$$

Where H(t), P, Rm and γ represent cumulative hydrogen production (mL), hydrogen-production potential (mL), maximum hydrogen-production rate (mL/h) and lag time (h), respectively. Parameters (P, Rm and γ) were estimated using the genetic algorithm. hydrogen production rate (Rm) was normalized with respect to working volume and defined as Rm/V $_{Media}$ (mL H_2/h L).

RESULTS AND DISCUSSIONS

In order to investigate the effect of external oxido- reduction potential on bio-hydrogen production, 9 experiments were carried out at voltage (0-800 mV) with two replications. The maximum hydrogen production rate and Accumulative hydrogen were calculated by the modified Gompertz equation presented in Table 1.

Table 1. Comparison of modified Gompertz equation parameters at different voltages

Sample	H_m(ml)	R_m(ml/lh)	γ(h)	R-square	Yield (mole H_2/mole Glucose)
Control	422.7	85.36	6.08	0.99	1.46
Anode$_{100mv}$	448	96.1	6.15	0.99	1.49
Anode$_{200mv}$	470	112.4	6.03	0.99	1.61
Anode$_{300mv}$	498	118.5	5.72	0.98	1.73
Anode$_{400mv}$	510	127.3	6.14	0.99	1.92
Anode$_{500mv}$	540	135.5	6.03	0.99	2.13
Anode$_{600mv}$	580	148.1	5.83	0.98	2.46
Anode$_{700mv}$	563	140.4	6.1	0.99	2.37
Anode$_{800mv}$	545	132.5	6.12	0.99	2.15

Figure 2 presents the effect of voltage on biohydrogen in cathode and anode cells. As can be seen in figure 2, the biohydrogen production rate in the anode is increased with the increase of voltage to 600 mV and at higher voltages, a decrease in hydrogen production rate is observed. Raise of voltage in the anode (raise of oxidation potential) can accelerate biochemical oxidation reactions involved in biohydrogen production and hence increase the biohydrogen production rate. According to table 2, increasing voltage to 600 mV leads to an increase of biohydrogen production in anode rate by 73% while biohydrogen production rate in cathode decreases which shows the negative effect of reduction potential on biohydrogen production rate.

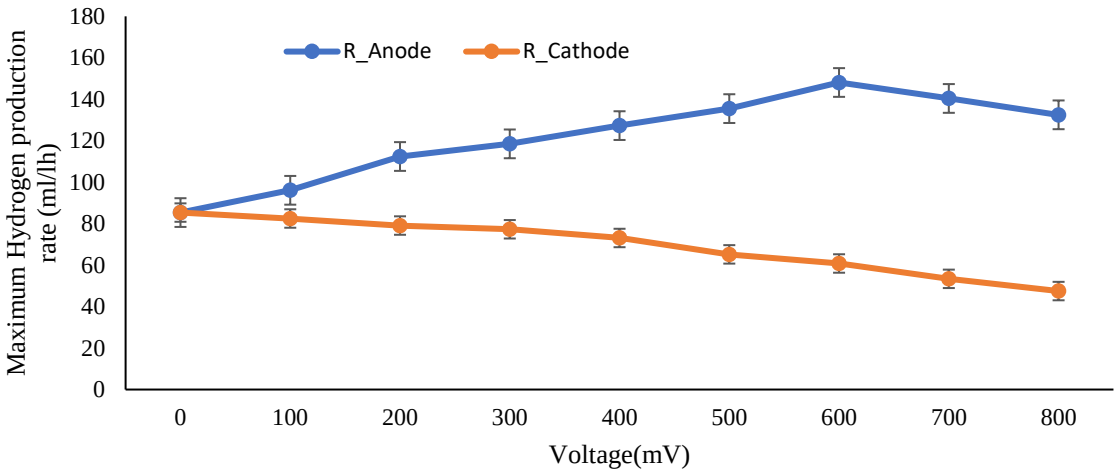

Figure 2. Maximum hydrogen production rate in anode and cathode as a function of voltage

The lag times variations based on voltage changes in anode and cathodes were shown in figure 3. As can be observed in figure 3, voltage changes do not have a considerable effect on lag times in both the anode and cathode.

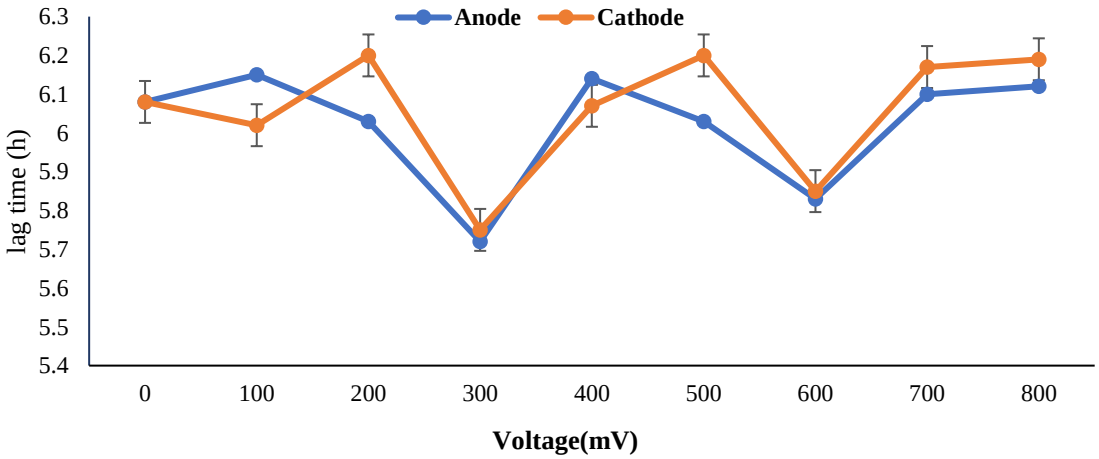

Figure 3. Lag time in anode and cathode as a function of voltage

The effect of voltage variations on yield in the anode and cathode was illustrated in figure 4. As can be seen, an increase of voltage to 600 mV leads to an increase of hydrogen yield in the anode by 68% as compared to the sample which implies the positive effect of oxidation potential raise on hydrogen production. Since some biohydrogen production end products like lactate and ethanol are produced to supply the NAD^+ required for the resumption of the glycolysis pathway, NADH oxidation to NAD^+ can block these undesired end products pathways and shift the process to the hydrogen production pathway. At voltages higher than 600 mV, hydrogen yield was decreased which might be due to disruption of optimum $NADH/NAD^+$. Although in the presence of NADH pyruvate reduced to lactate and ethanol and hence hydrogen yield decreased, a part of total hydrogen can be produced as a result of NADH oxidation at appropriate conditions (Hallenbeck, 2009). Thus, regulation of $NADH/ NAD^+$ is one of the most important parameters for the biohydrogen production process. Despite of Anode, hydrogen yield in the cathode was decreased with the increase of voltage, so it can be concluded that the increase

of reduction potential directed the biochemical reactions to produce undesired end products and hence less hydrogen will be produced. The soluble metabolites analyses can help us to study the process more comprehensively. Soluble metabolite distribution in the effluent was demonstrated in table 2 and the effect of voltage on metabolite distribution in the anode was illustrated in figure 5. According to table 2, the acetate percentage in effluent increased from 21.3% to 46.9% when the voltage increased to 600 mV which indicates that the-raise of oxidation potential, motivates the acetate production pathway. Since the acid-forming pathway with acetic acid as a major metabolite dominated the metabolic flow during the H_2 production (Venkata Mohan et al., 2007), the positive effect of oxidation potential on bio-hydrogen production can be explained. The butyrate and butanol percentages in effluent were almost constant and the ethanol percentage was reduced by 50% approximately with the increase of voltage to 600 mV. Since hydrogen is not produced in the ethanol production pathway (Guo et al., 2010), a decrease in ethanol percentage can be another reason for the increase of biohydrogen yield with the increase of oxidation potential. These results were the same of Cai et all (2013) results which concluded that ethanol pathway blocking leads to an increase of hydrogen production by 20%. Also, it was observed that the lactate distribution in the effluent decreased from 18.3% to 3.7% with the raise of voltage to 600 mV. Increasing oxidation potential in the anode resulted in more NADH oxidation there pyruvate reduction to lactate is prevented and more acetate and hydrogen are produced (Morimoto et al., 2005).

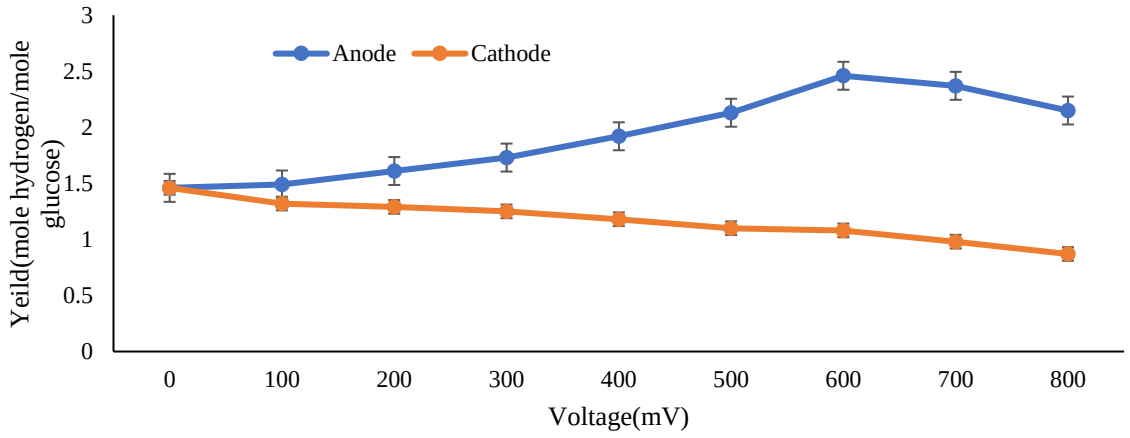

Figure 4. Yield in Anode and Cathode as a function of voltage

Table2. Comparison of metabolite distribution in anode

Sample	Acetate (%)	Butyrate (%)	Acetone (%)	Ethanol (%)	Butanol (%)	Lactate (%)	A/B
Control	21.3	25.9	2.6	13.2	13.7	18.3	0.82
Anode$_{100mV}$	27.5	29.9	2.7	12.6	13.1	15.2	0.91
Anode$_{200mV}$	32.7	29.4	1.9	11.7	12.8	11.5	1.11
Anode$_{300mV}$	39.4	29.5	1.6	9.8	12.4	7.3	1.33
Anode$_{400mV}$	43.2	29.1	1.3	9.1	11.1	6.2	1.48
Anode$_{500mV}$	44.8	29.6	1.1	8.7	11.6	4.2	1.51
Anode$_{600mV}$	46.9	20.8	1.0	6.2	11.4	3.7	1.52
Anode$_{700mV}$	43.4	33.5	1.3	6.4	11.5	3.9	1.30
Anode$_{800mV}$	41.3	34.8	1.6	6.7	11.3	3.4	1.18

According to table 2, the acetate to butyrate ratio (A/B ratio) was increased from 0.82 to 1.52 as a result of a voltage increment to 600 mV. This was consistent with higher yields and production rates of Hydrogen which in turn indicates that a higher A/B ratio could result in higher hydrogen productivity. This may be explained by the following equations:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (3)$$

Equation 2 and equation 3 consider the major metabolic pathway involved in hydrogen production. From these equations, it might be concluded that with the increase of oxidation potential, the reaction is driven predominantly toward acetate production resulting in higher production of hydrogen. This is in close agreement with previous reports (Annous et al., 1996; S. H. Kim et al., 2006) claiming that the higher A/B ratio facilitates hydrogen production.

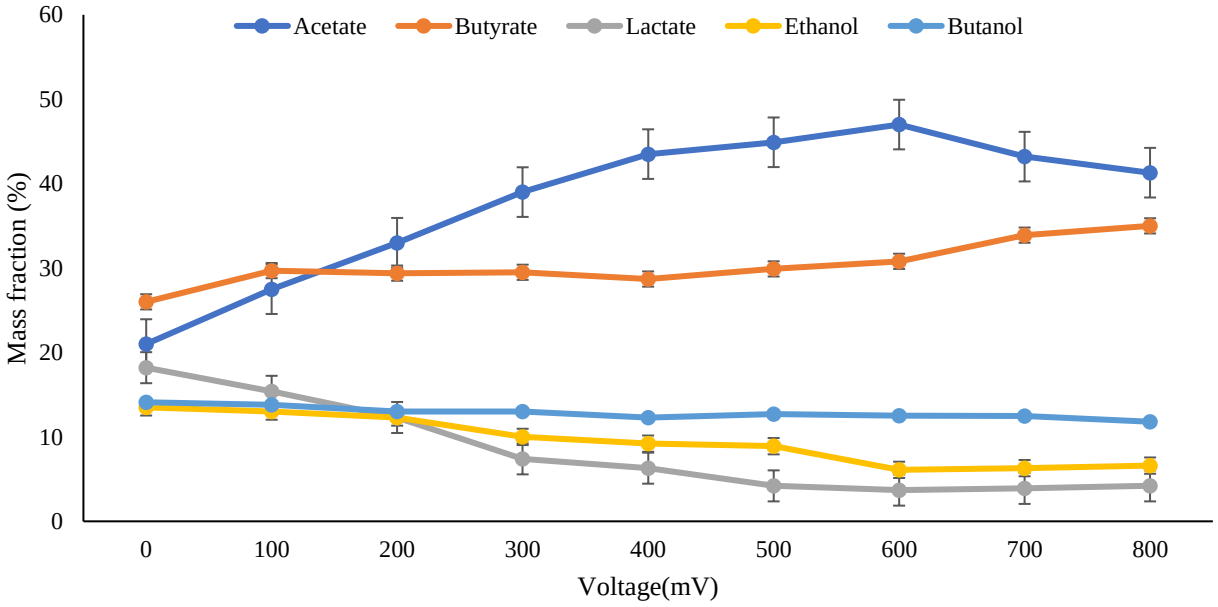

Figure 5. Metabolite distribution in the effluent as a function of voltage

CONCLUSION

The effect of oxidation potential on dark fermentation production of biohydrogen was investigated. Based on the observed results, raise of voltage to 600 mV leads to increases in hydrogen production rate and yield in the anode (oxidation half-cell) by 73% and 67% respectively as a result of undesired metabolites like ethanol and lactate decrement in the effluent. In the cathode biohydrogen production yield would be decreased with voltage raising. $NADH/NAD^+$ ratio is an efficient parameter for biohydrogen production that could be influenced by external oxidation-reduction potential in order to regulate and prepare for usage in the microbial metabolic pathway.

REFRENCES

Annous, B. A., Shieh, J. S., Shen, G. J., Jain, M. K., & Zeikus, J. G. (1996). Regulation of hydrogen metabolism in Butyribacterium methylotrophicum by substrate and pH. *Applied Microbiology and Biotechnology*, *45*(6), 804–810. https://doi.org/10.1007/s002530050766

Antonopoulou, G., Ntaikou, I., Stamatelatou, K., & Lyberatos, G. (2011). Biological and fermentative production of hydrogen. *Handbook of Biofuels Production: Processes and Technologies*, 305–346. https://doi.org/10.1533/9780857090492.2.305

Arimi, M. M., Knodel, J., Kiprop, A., Namango, S. S., Zhang, Y., & Geißen, S. U. (2015). Strategies for improvement of biohydrogen production from organic-rich wastewater: A review. *Biomass and Bioenergy*, *75*, 101–118. https://doi.org/10.1016/j.biombioe.2015.02.011

Balat, M. (2009). Production of Hydrogen via Biological Processes. *9*, *31*(20), 1802–1812. https://doi.org/10.1080/15567030802463109

Bisaillon, A., Turcot, J., & Hallenbeck, P. C. (2006). The effect of nutrient limitation on hydrogen production by batch cultures of Escherichia coli. *International Journal of Hydrogen Energy*, *31*(11), 1504–1508. https://doi.org/10.1016/j.ijhydene.2006.06.016

Cai, G., Jin, B., Monis, P., & Saint, C. (2013). A genetic and metabolic approach to redirection of biochemical pathways of Clostridium butyricum for enhancing hydrogen production. *Biotechnology and Bioengineering*, *110*(1), 338–342. https://doi.org/10.1002/bit.24596

Cao, Y., Liu, H., Liu, W., Guo, J., & Xian, M. (2022). Debottlenecking the biological hydrogen production pathway of dark fermentation: insight into the impact of strain improvement. *Microbial Cell Factories*, *21*(1), 1–16. https://doi.org/10.1186/s12934-022-01893-3

Das, D., & Veziroğlu, T. N. (2001). Hydrogen production by biological processes: a survey of literature. *International Journal of Hydrogen Energy*, *26*(1), 13–28. https://doi.org/10.1016/S0360-3199(00)00058-6

Dzulkarnain, E. L. N., Audu, J. O., Wan Dagang, W. R. Z., & Abdul-Wahab, M. F. (2022). Microbiomes of biohydrogen production from dark fermentation of industrial wastes: current trends, advanced tools and future outlook. *Bioresources and Bioprocessing 2022 9:1*, *9*(1), 1–25. https://doi.org/10.1186/s40643-022-00504-8

Elam, C. C., Padró, C. E. G., Sandrock, G., Luzzi, A., Lindblad, P., & Hagen, E. F. (2003). Realizing the hydrogen future: the International Energy Agency's efforts to advance hydrogen energy technologies. *International Journal of Hydrogen Energy*, *28*(6), 601–607. https://doi.org/10.1016/S0360-3199(02)00147-7

Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P. N. L., & Esposito, G. (2015). A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products. *Applied Energy*, *144*, 73–95. https://doi.org/10.1016/j.apenergy.2015.01.045

Guo, X. M., Trably, E., Latrille, E., Carrre, H., & Steyer, J. P. (2010). Hydrogen production from agricultural waste by dark fermentation: A review. *International Journal of Hydrogen Energy*, *35*(19), 10660–10673. https://doi.org/10.1016/j.ijhydene.2010.03.008

Hallenbeck, P. C. (2009). Fermentative hydrogen production: Principles, progress, and prognosis. *International Journal of Hydrogen Energy*, *34*(17), 7379–7389. https://doi.org/10.1016/j.ijhydene.2008.12.080

Hallenbeck, P. C., & Ghosh, D. (2012). Improvements in fermentative biological hydrogen production through metabolic engineering. *Journal of Environmental Management*, *95*(SUPPL.), S360–S364. https://doi.org/10.1016/j.jenvman.2010.07.021

Jeong, T. Y., Cha, G. C., Yeom, S. H., & Choi, S. S. (2008). Comparison of hydrogen production by four representative hydrogen-producing bacteria. *Journal of Industrial and Engineering Chemistry*, *14*(3), 333–337. https://doi.org/10.1016/j.jiec.2007.09.014

Kim, D. H., Kim, S. H., Kim, H. W., Kim, M. S., & Shin, H. S. (2011). Sewage sludge addition to food waste synergistically enhances hydrogen fermentation performance. *Bioresource Technology*, *102*(18), 8501–8506. https://doi.org/10.1016/j.biortech.2011.04.089

Kim, S. H., Han, S. K., & Shin, H. S. (2006). Effect of substrate concentration on hydrogen production and 16S rDNA-based analysis of the microbial community in a continuous fermenter. *Process Biochemistry*, *41*(1), 199–207. https://doi.org/10.1016/j.procbio.2005.06.013

Konieczny, A., Mondal, K., Wiltowski, T., & Dydo, P. (2008). Catalyst development for thermocatalytic decomposition of methane to hydrogen. *International Journal of Hydrogen Energy*, *33*(1), 264–272. https://doi.org/10.1016/j.ijhydene.2007.07.054

Koutra, E., Tsafrakidou, P., Sakarika, M., & Kornaros, M. (2020). Microalgal Biorefinery.

Microalgae Cultivation for Biofuels Production, 163–185. https://doi.org/10.1016/B978-0-12-817536-1.00011-4

Levin, D. B., Zhu, H., Beland, M., Cicek, N., & Holbein, B. E. (2007). Potential for hydrogen and methane production from biomass residues in Canada. *Bioresource Technology*, *98*(3), 654–660. https://doi.org/10.1016/j.biortech.2006.02.027

Li, R., Pei, S., Chen, B., Song, Y., Zhang, T., Yang, W., & Shaman, J. (2020). Substantial undocumented infection facilitates the rapid dissemination of novel coronavirus (SARS-CoV-2). *Science*, *368*(6490), 489–493. https://doi.org/10.1126/science.abb3221

Liu, H., Cheng, T., Xian, M., Cao, Y., Fang, F., & Zou, H. (2014). Fatty acid from the renewable sources: a promising feedstock for the production of biofuels and biobased chemicals. *Biotechnology Advances*, *32*(2), 382–389. https://doi.org/10.1016/j.biotechadv.2013.12.003

Morimoto, K., Kimura, T., Sakka, K., & Ohmiya, K. (2005). Overexpression of a hydrogenase gene in Clostridium paraputrificum to enhance hydrogen gas production. *FEMS Microbiology Letters*, *246*(2), 229–234. https://doi.org/10.1016/j.femsle.2005.04.014

Shaterzadeh, M. J., & Ataei, S. A. (2017). The effects of temperature, initial pH, and glucose concentration on biohydrogen production from Clostridium acetobutylicum. *39*(11), 1118–1123. https://doi.org/10.1080/15567036.2017.1297875

Singh, N., & Sarma, S. (2022). Biological routes of hydrogen production: a critical assessment. *Handbook of Biofuels*, 419–434. https://doi.org/10.1016/B978-0-12-822810-4.00021-X

Venkata Mohan, S., Vijaya Bhaskar, Y., & Sarma, P. N. (2007). Biohydrogen production from chemical wastewater treatment in biofilm configured reactor operated in periodic discontinuous batch mode by selectively enriched anaerobic mixed consortia. *Water Research*, *41*(12), 2652–2664. https://doi.org/10.1016/j.watres.2007.02.015