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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

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ARTICLE INFO

Article type:

Research Article

Article history:

Received 23 September 2023

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 causing 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 fermented 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>



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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). Diminution of fossil fuels reserves, and emission of greenhouse gases and pollutants like CO_x, NO_x, SO_x, C_xH_y, and ash, have inspired 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; Bisailon 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 remediation (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, CO₂, alcohols and H₂ 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 H₂/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 H₂ 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 H₂ 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 H₂ 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 H₂ production yield by 37% compared to the wild-type strain (Bisailon 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, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25, K_2HPO_4 1, KH_2PO_4 1, and L-cysteine-HCl.H₂O 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, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25, K_2HPO_4 1, KH_2PO_4 1, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.025, and L-cysteine-HCl.H₂O 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.

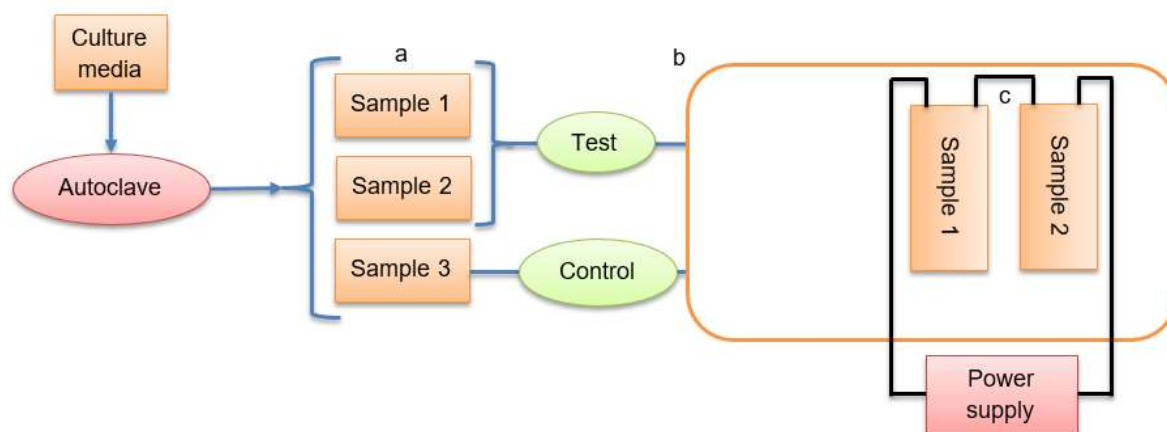


Figure 1. a) 10% of master culture (inoculant) added to each Erlenmeyer, b) incubation zone 35°C, c) salt bridge KCl 2M and Agar, d) cathode (reduction), e) anode (oxidation)

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 N₂ 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:

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

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

Sample	H _m (ml)	R _m (ml/h)	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

Where H(t), P, R_m and γ represent cumulative hydrogen production (mL), hydrogen-production potential (mL), maximum hydrogen-production rate (mL/h) and lag time (h), respectively. Parameters (P, R_m and γ) were estimated using the genetic algorithm. hydrogen production rate (R_m) was normalized with respect to working volume and defined as R_m/V_{Media} (mL H₂/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.

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.

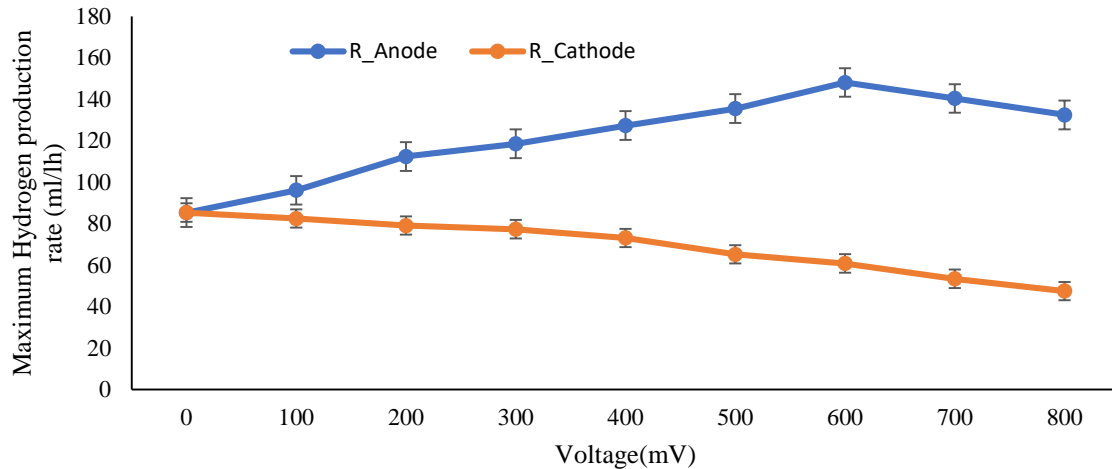


Figure 2. Maximum hydrogen production rate in anode and cathode as a function of voltage

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.

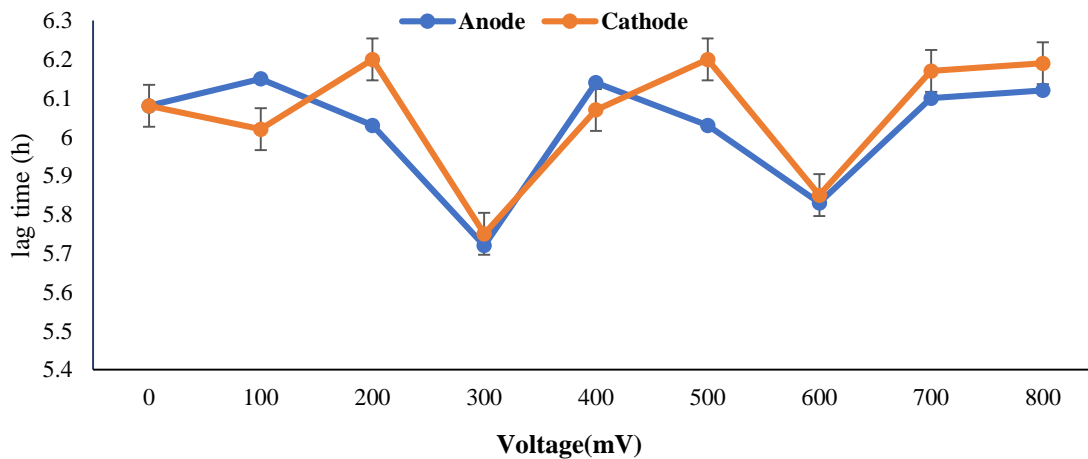


Figure 3. Lag time in anode and cathode as a function of voltage

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 H₂ 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 al (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).

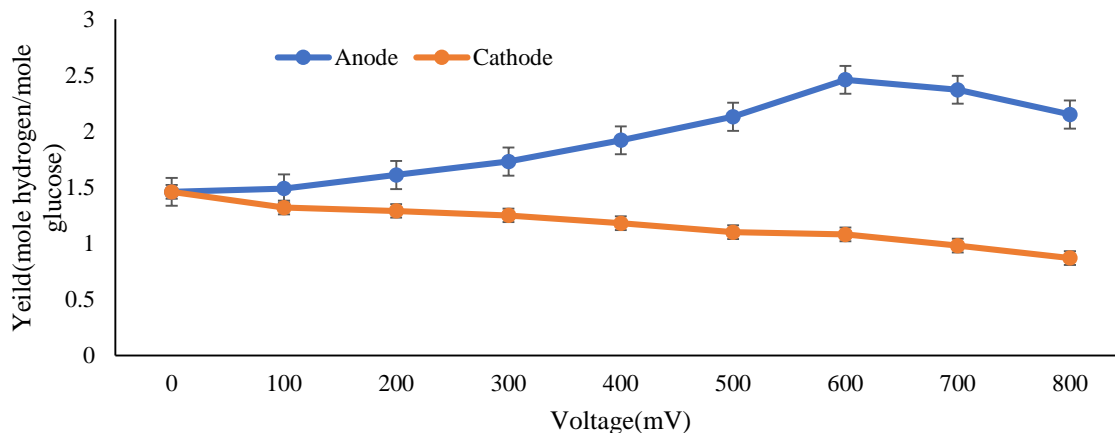
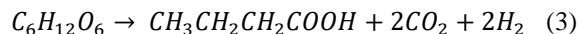
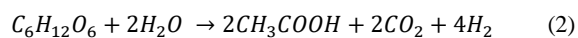


Figure 4. Yield in Anode and Cathode as a function of voltage

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:



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.

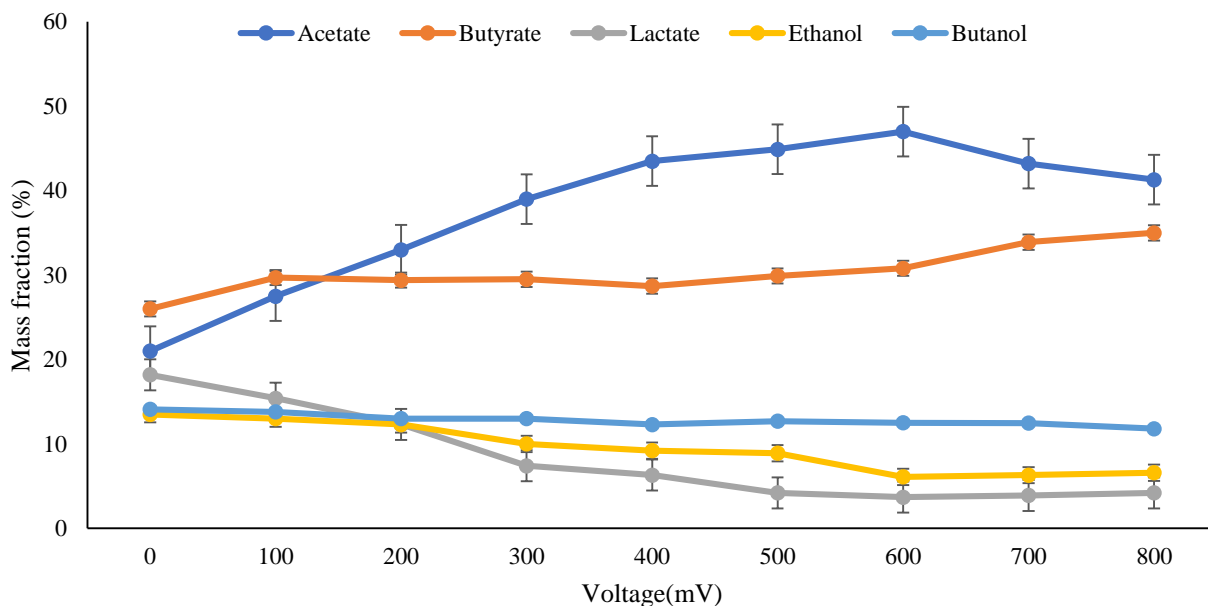


Figure 5. Metabolite distribution in the effluent as a function of voltage

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.

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