Original Article

Comparison of Acetate-butyrate and Acetate-ethanol Metabolic Pathway in Biohydrogen Production

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Abstract

Background: Hydrogen gas is the cleanest energy carrier and could be produced by biological process. Dark fermentation is one of the biohydrogen production methods that carried out just on organic wastes conversion. **Methods:** In this study, the batch tests were conducted to compare the biohydrogen production and glucose fermentation via acetate-butyrate and acetate-ethanol metabolic pathway induced by NaOH and KOH (10 M) pretreatment. In batch test, the glucose concentration in the feed was varied from 3.75 to 15 g/L under mesophilic conditions (37°C ± 1°C). In order to sludge pretreatment, NaOH and KOH (as an alkaline agent) was used. **Results:** Batch tests showed that maximum biohydrogen production under NaOH (2.7 ± 0.5 L) and KOH (2.2 ± 0.7 L) pretreatment was achieved at 15 g/L of influent glucose. In the batch test, with increasing influent glucose concentration, the lower yields of hydrogen were observed. The biohydrogen reactions had good electron closure (5.2%–13.5%) for various glucose concentrations and pretreatments. For NaOH and KOH pretreatment, the biohydrogen yield decreased from 2.49 to 1.63 and from 2.22 to 1.2 mol H_2/mol glucose, respectively, when glucose concentration increased from 3.75 to 15 g/L. **Conclusions:** By applying alkaline sludge pretreatment by NaOH and KOH, the glucose fermentation was followed with acetate-butyrate and acetate-ethanol metabolic pathway, respectively. The lower biohydrogen yields were observed under acetate-ethanol metabolic pathway and related to metabolically unfavorable for biohydrogen production.

Keywords: *Acetate-butyrate pathway, acetate-ethanol metabolic, biohydrogen production*

Introduction

The concerns of environmental issues have proven hydrogen as an alternative fuel because of its nonpolluting features.[1,2] Biohydrogen production is possible by nonbiological and biological methods. The biological method for hydrogen production includes direct photobiological production, indirect photobiological production, photo fermentation, and dark fermentation.[3]

Dark fermentation is one of the biohydrogen production methods that carried out independently on fuel energy and just on organic wastes conversion. In using the sludge as a mix culture for biohydrogen production, pretreatment is necessary for deactivation of methane-producing bacteria. Different methods such as acid, base, heating, using chemical compound, aeration, and ultrasonication were used as a pretreatment for enriching biohydrogen-producing bacteria.[4-7]

The method that was used for sludge pretreatment is an effective factor in the dominance of special pathway for biohydrogen production, for example, by heating sludge as a pretreatment method can select spore-forming bacteria such as clostridia or pretreatment by aeration led to *Clostridium* sp. and *Enterobacter* dominance.[6,8] The efficiency of biohydrogen production process is different depending on pretreatment method, dominance bacteria, substrate, and metabolic pathway.[9,10] With understanding metabolic pathway, the calculation of theoretical hydrogen yield is possible.[11]

The main introduced pathways for hydrogen production in dark fermentation are acetate-butyrate and acetate-ethanol pathway.[2,12] Acetate-propionate pathway is another fermentation pathway that does not produce any hydrogen.[8]

Acetate-butyrate metabolic has been reported in many studies as a dominant pathway for hydrogen production that has been down by butyrate type fermentation bacteria such as *Clostridium*, *Butyrivibrio*, and *Bacillus*, but this pathway can

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convert to butanol production way that is the hydrogen consumption pathway. In comparison to acetate-butyrate metabolic pathway, acetate-ethanol pathway is more stable way for hydrogen production and it seems that this pathway has been down by *Ethanoligenens*, *Acetanaerobacterium*, *Clostridum*, *Rhodopseudomona*, and *Citrobacter* (the dominant genera are unknown).[8,12]

Different pretreatment methods lead to the domination of different bacterial communities in biohydrogen production and can face with different metabolic pathways. Production of various components in each metabolic pathway shows the different distributions of electron equivalents. Hence, in this study, biohydrogen production via acetate-butyrate and acetate-ethanol pathway induced by sludge pretreatment was studied and also biohydrogen production stoichiometry was carried out. In addition, for better understanding of the microbial metabolism, the mass balance recovery was down.

Materials and Methods

Enriching of biohydrogen-producing inoculum

The parent anaerobic digested sludge was extracted from a full-scale municipal sludge digester (South Municipal Wastewater Treatment Plant, Tehran, Iran). The properties of parent anaerobic sludge are summarized in Table 1. According to Amin *et al*.'s study, this anaerobic sludge showed good biohydrogen production potential.[9] Before alkaline pretreatment, the sludge was sifting by a standard sieve #16 with 1.19 mm pore size. Two different alkaline pretreatment agents were evaluated and are including NaOH and KOH agents (10 M solution). In order to biohydrogen-producing bacteria enrichment, the solution pH of anaerobic sludge was increased to 12 using each alkaline agent individually under anaerobic environment for 24 h and then sludge pH adjusted to 7 by HCl.[13]

Table 1: Properties of parent anaerobic sludge

Parameter	Unit	Value
pH	-	7.75±0.1
Soluble COD	g/L	2.5±0.4
Total COD	g/L	12.6±2.2
VSS	g/L	16.84±3.4
And TSS	g/L	32.56±6.6

COD – Chemical oxygen demand; VSS – Volatile suspended solid; TSS – Total suspended solid

Batch test procedure

The batch tests were carried out in 500 mL glass bottles that contain 200 mL feed solution and 200 mL of pretreated sludge as demonstrated by Amin *et al*.'s study.[9] Glucose was used as sole carbon source at influent concentration of 3.75, 7.5, 11.25, and 15 g/L that is equal to 0.5, 1, 1.5, and 2 electron equivalents (e^- eq), respectively. The details of medium composition are described in Amin *et al*.'s study.[14] The batch tests were done in duplicate and incubated at 37°C ± 1°C and stirred glass flasks (360 s idle and 30 s mixing) for 48 h. Before incubation, for insurance of anaerobic condition and sludge and substrate contact, each vial was purged by N_2 gas for 3 min (400 mL/min).

Analytical methods

The fermentation metabolites including volatile fatty acids (VFAs) such as acetic, propionic, butyric, and valeric acid and solvents including methanol, ethanol, and acetone were analyzed by a flame ionization detector (GC-FID, Agilent 7890A GC with Varian CP-Sil5cb column) as described in the literature.[15,16] The chromatographic program was as follows: the helium gas at flow rate of 1 mL/min (19.086 cm/s) was used as a carrier gas; oven temperature was 70°C (3 min), first ramp as 10°C/min to 130°C (0 min), second ramp as 5°C/min to 180°C (5 min), and post run 250°C (1 min). The nitrogen gas was used as a makeup at flow rate of 30 mL/min. The standard curve of VFAs and solvent is shown in Figure 1. Other test methods including solution pH, alkalinity, COD, and glucose residual were measured using a glass body pH probe (CG 824 SCHOTT), titration method, closed reflux, colorimetric method, and phenol-sulfuric acid methods according to Amin *et al*.'s study.[9]

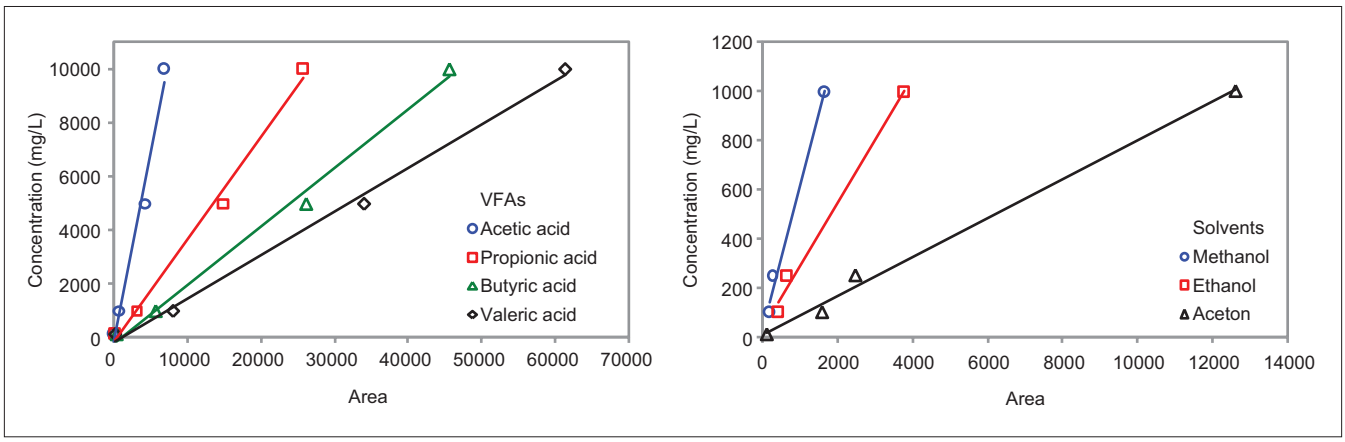

Figure 1: Volatile fatty acids and solvent standard curve

Calculation

For establishing the mass balance recovery based on the e^- eq in the batch tests, Eq. 1 was used as proposed in previous literature.[17,18]

$$e^-_{glu,in} = e^-_{SEP} + e^-_{H_2} + e^-_{biomass} + e^-_{glu,eff} \quad (1)$$

Where $e^-_{glu,\ in}$ is e^- eq of influent glucose, e^-_{SEP} is e^- eq of soluble end products, $e^-_{H_2}$ is e^- eq of biohydrogen during incubation period, $e^-_{biomass}$ is e^- eq of biomass growth, and $e^-_{glu,eff}$ is the e^- eq of residual glucose after incubation. The SEPs include acetate, propionate, butyrate, formate, lactate, acetone, methanol, and ethanol. The conversion of e^- eq measured value was done based on this fact 1 e^- eq is equal to 7.5 g of glucose, 7.38 g of acetate, 5.22 g of propionate, 4.35 g of butyrate, 22.65 g of formate, 7.42 g of lactate, 5.34 g of methanol, 3.84 g of ethanol, and 5.46 g of biomass.[19]

Results

As shown in Figure 2, in case of KOH pretreatment, the biohydrogen production was 0.15 ± 0.05 L, 0.65 ± 0.12 L, 1.2 ± 0.2 L, and 2.2 ± 0.7 L for 3.75, 7.5, 11.25, and 15 g/L of influent glucose, respectively. Compared with the KOH pretreatment, as the NaOH was used as pretreatment agent, the biohydrogen production was enhanced to 0.2 ± 0.05 L, 0.75 ± 0.1 L, 1.5 ± 0.3 L, and 2.7 ± 0.5 L for 3.75, 7.5, 11.25, and 15 g/L of influent glucose, respectively.

The glucose conversion efficiency during biohydrogen production with NaOH and KOH pretreatment of anaerobic sludge is depicted in Figure 3.

At glucose concentration of 3.75, 7.5, 11.25, and 15 g/L, the glucose conversion was 90.7% ± 0.05%, 94.1% ± 0.1%, 93 ± 0.3, and 96% ± 0.5% for NaOH pretreatment and also 89.7% ± 0.05%, 92.9% ± 0.1%, 93.8% ± 0.2%, and 93.5% ± 0.6% for KOH pretreatment, respectively.

Tables 2 and 3 summarize the fractions of electron acceptor at different glucose concentrations under NaOH and KOH sludge pretreatment. As depicted in Tables 2 and 3, the dominate electron acceptors under NaOH and KOH pretreatment were acetate and butyrate and also ethanol and acetate, respectively.

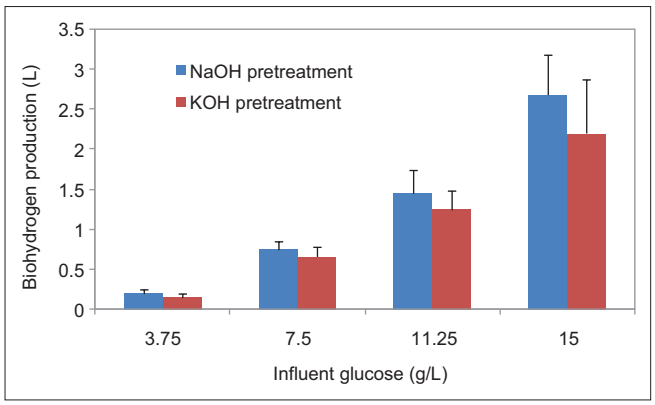

Figure 2: Average biohydrogen production during batch tests

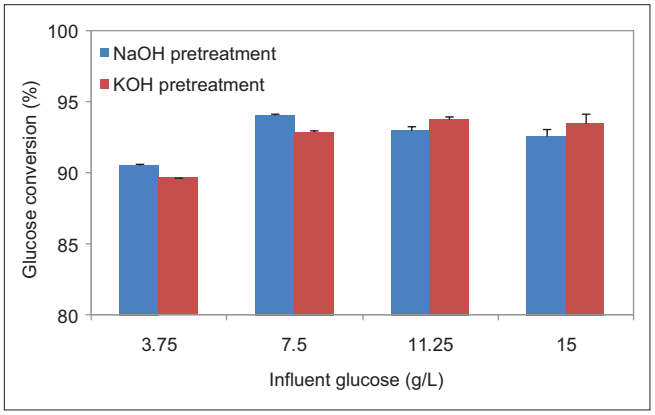

Figure 3: Glucose conversion during biohydrogen production

Table 2: Fractions of electron acceptor under NaOH sludge pretreatment

Compounds	Glucose concentration			
	3.75 (g/L) (%)	7.5 (g/L) (%)	11.25 (g/L) (%)	15 (g/L) (%)
Glucose influent	200 (100)	400 (100)	600 (100)	800 (100)
Acetate	74.3±2.1 (40.1)	140.3±8.1 (36.3)	194.5±7.7 (34.5)	248.7±11.1 (33.6)
Propionate	1.8±3.1 (1.1)	9.4±1.1 (2.4)	9.9±2.1 (1.7)	9±1.8 (1.3)
Butyrate	57.8±3.1 (31.2)	101.7±5.1 (26.3)	193.5±5.1 (34.3)	230.3±10.1 (31.1)
Formate	ND	ND	ND	ND
Lactate	ND	ND	ND	ND
Acetone	ND	ND	ND	ND
Methanol	ND	ND	ND	ND
Ethanol	ND	ND	ND	ND
Biomass	16.9±1.9 (9.1)	56.7±2.3 (14.6)	27.7±4.5 (4.9)	27.7±3.2 (3.7)
Residual glucose	18.9±8.9 (10.2)	25.4±5.4 (6.6)	42.0±10.1 (7.4)	42.0±9.8 (5.7)
Biohydrogen	15.7±4.9 (8.4)	53.4±6.8 (13.8)	97.3±11.1 (17.2)	182±13.1 (24.6)
Total	182.5	387.1	564.6	740.7
Δe^- eq (%)	8.7	3.1	5.8	7.3

*Units are in e^- eq (%). e^- eq – Electron equivalent; ND – Non Detectable

The final form of stoichiometric reactions of biohydrogen production under NaOH and KOH pretreatment as function of influent glucose concentration is shown in Table 4.

Discussion

Biohydrogen production and glucose conversion

Overall, the analysis of produced biogas was depicted that biogas contains 40%–44% of hydrogen and no methane was detected, which demonstrated efficient inactivation of methanogens bacteria during sludge pretreatment. The amounts of biohydrogen production during batch tests are shown in Figure 2. As shown in Figure 2, in overall, with increasing influent glucose concentration, the amount of biohydrogen production increased, suggesting that the glucose concentration had a great effect on biohydrogen production. The highest biohydrogen production was obtained at 15 g/L of influent glucose concentration with respect to 2.6 ± 0.5 L and 2.2 ± 0.7 L for NaOH and KOH pretreatment, respectively.

The obtained results are in line with previous study. According to the report by Van Ginkel *et al*., with increasing influent COD from 0.5 to 20 g/L, the biohydrogen production increases from 0.1 to 2.8 L.[20]

Overall, the glucose conversion efficiency during batch test was high and glucose conversion was more than 90%. With application of KOH as a pretreatment agent, the glucose conversion was slightly lower than NaOH pretreatment and related to dominant biohydrogen production bacteria. The glucose conversion is responsible for complete fermentation and it has been demonstrated that the culture media are highly capable of glucose fermentation and biohydrogen production. In the previous study, Whang *et al*. reported 99% of the glucose consumption efficiency.[11]

End products and mass balance recovery

Depending on initial microorganisms culture and operation conditions, during carbohydrates fermentation, as the primary metabolites, several VFAs and alcohols are produced.[11] The end products of biohydrogen production included volatile organic acids (acetic, propionic, butyric, formic, and lactic acid), alcohols (methanol, ethanol, and acetone), biomass, biohydrogen gas, and also residual glucose. The distribution of glucose fermentation products in biohydrogen batch tests conducted with the NaOH pretreatment based on the e^- eq and percentage is summarized in Table 2.

Table 3: Fractions of electron acceptor under KOH sludge pretreatment

Compounds	Glucose concentration			
	3.75 (g/L) (%)	7.5 (g/L) (%)	11.25 (g/L) (%)	15 (g/L) (%)
Glucose influent	200 (100)	400 (100)	600 (100)	800 (100)
Acetate	24.4±8.1 (13.5)	78.6±11.1 (16)	138.2±21.1 (24.3)	165.3±18.1 (22.6)
Propionate	3.9±0.9 (2.1)	3.9±1.4 (1)	3.9±1.1 (0.7)	8.5±2.1 (1.2)
Butyrate	17.5±4.9 (9.7)	59.8±5.8 (15.8)	78.1±6.1 (13.8)	101.1±9.1 (13.8)
Formate	ND	ND	ND	ND
Lactate	ND	ND	ND	ND
Acetone	ND	ND	ND	ND
Methanol	ND	ND	ND	ND
Ethanol	79±12.1 (43.6)	121.8±12.2 (32.1)	205.1±16.1 (36.1)	226.1±13.1 (30.8)
Biomass	15.2±9.1 (8.4)	31.7±8.1 (8.4)	6.4±2.1 (1.1)	7.2±3.1 (1)
Residual glucose	20.7±3.1 (11.4)	28.3±4.8 (7.5)	37.5±4.1 (6.6)	51.8±9.1 (7.1)
Biohydrogen	12.1±6.8 (11.4)	54.9±10.1 (14.5)	69.1±9.1 (17.3)	127.2±12.1 (23.6)
Total	172.8	379	567.6	732.6
Δe^- eq (%)	13.5	5.2	5.3	8.3

*Units are in e^- eq (%). e^- eq – Electron equivalent; ND – Non Detectable

Table 4: Stoichiometric reactions of biohydrogen production under NaOH and KOH pretreatment

Pretreatment agent	Glucose concentration (g/L)	Overall stoichiometric reactions
NaOH	3.75	$C_6H_{12}O_6 + 5.22\ H_2O = 2.98\ C_2H_3O_2^- + 0.04\ C_3H_5O_2^- + 0.93\ C_4H_7O_2^- + 2.49\ H_2 + 3.92\ CO_2 + 5.42\ H^+$
	7.5	$C_6H_{12}O_6 + 1.41\ H_2O = 1.38\ C_2H_3O_2^- + 0.05\ C_3H_5O_2^- + 0.4\ C_4H_7O_2^- + 2.10\ H_2 + 1.77\ CO_2 + 2.63\ H^+$
	11.25	$C_6H_{12}O_6 + 3.07\ H_2O = 0.76\ C_2H_3O_2^- + 0.02\ C_3H_5O_2^- + 0.30\ C_4H_7O_2^- + 1.78\ H_2 + 3.21\ CO_2 + 1.58\ H^+$
	15	$C_6H_{12}O_6 + 3.99\ H_2O = 0.56\ C_2H_3O_2^- + 0.01\ C_3H_5O_2^- + 0.21\ C_4H_7O_2^- + 1.63\ H_2 + 4.03\ CO_2 + 1.17\ H^+$
KOH	3.75	$C_6H_{12}O_6 + 3.23\ H_2O = 1.07\ C_2H_3O_2^- + 0.1\ C_3H_5O_2^- + 0.31\ C_4H_7O_2^- + 2.30\ C_2H_5OH + 2.12\ H_2 + 2.25\ CO_2 + 3.56\ H^+$
	7.5	$C_6H_{12}O_6 + 1.71\ H_2O = 0.75\ C_2H_3O_2^- + 0.02\ C_3H_5O_2^- + 0.23\ C_4H_7O_2^- + 0.77\ C_2H_5OH + 1.96\ H_2 + 2.19\ CO_2 + 1.97\ H^+$
	11.25	$C_6H_{12}O_6 + 3.3\ H_2O = 0.53\ C_2H_3O_2^- + 0.01\ C_3H_5O_2^- + 0.12\ C_4H_7O_2^- + 0.52\ C_2H_5OH + 1.5\ H_2 + 2.82\ CO_2 + 1.34\ H^+$
	15	$C_6H_{12}O_6 + 4.18\ H_2O = 0.37\ C_2H_3O_2^- + 0.01\ C_3H_5O_2^- + 0.09\ C_4H_7O_2^- + 0.33\ C_2H_5OH + 1.2\ H_2 + 4.23\ CO_2 + 0.98\ H^+$

In case of NaOH pretreatment [Table 2], dominate soluble end products were acetate and butyrate and supported the acetate-butyrate pathway fermentation.[17,21] The high acetate production suggested that the fermenting bacteria could efficiently conserve energy through acetate production.[17,22] In all batch tests, the portion of biohydrogen was fluctuated from 11% to 23% and the little portion of electron sink was related to propionate (<2%). Formate, lactate, acetone, methanol, and ethanol were not detected. In batch tests, the lactate was not observed and it has been demonstrated that the generation of molecular hydrogen by disposal mechanisms of protons and electrons is not blocked.[23]

Table 3 summarizes the end metabolites produced in batch tests with KOH sludge pretreatment. The amount and characteristics of soluble end products were highly related to influent glucose.

The sum of VFAs and ethanol kept increasing with influent glucose and reached to 500 e^- eq when influent glucose was 15 g/L [Table 3]. Among metabolites, ethanol is a major soluble end product found in the studied influent glucose. The ethanol portion was 43.6%, 32.1%, 36.1%, and 30.8% of e^- eq for 3.75, 7.5, 11.25, and 15 g/L of influent glucose, respectively. The results supported our hypothesis about ethanol-based biohydrogen-producing bacteria.[8,24]

The e^- eq of biohydrogen was as high as 11.4%, 14.5%, 17.3%, and 23.6% of end products for 3.75, 7.5, 11.25, and 15 g/L of influent glucose, respectively. The e^- eq portion of propionate was low and ranged from 0.7% to 2.1%. As the previous study demonstrated that formation of reduced ferredoxin was a critical step for biological hydrogen production, propionate, formate, and lactate accumulation had led to the hydrogen production reduction.[17,25]

For all tests, the related e^- eq biomass ranged between 1% to 9.1%, which was lower than those reported by Liu *et al*., but in the range of Lee *et al*. and Amin *et al*.'s study.[9,21,25] These results demonstrated that actually growing of cells and adenosine triphosphate synthesis.[26] For all batch tests, the mass balance recovery based on the e^- eq were closed within 5.2%–13.5% and depicted good detection of end products.

The dominance component that produced by NaOH treated culture was butyric and acetic acid that shown the acetic-butyric pathway for hydrogen production. For KOH pretreated culture, the possible pathway was acetic-ethanol, because in all glucose concentrations, the major products were acetic and ethanol. It was concluded that different pretreatment methods not only have different efficiencies for methane-producing bacteria but also can choose different hydrogen production communities by various metabolic pathways.[13]

Stoichiometry reaction

The stoichiometry reaction construction was carried out according to a proposed method by Lee *et al*.[18] The half-reactions for electron acceptor and donor were chosen from literature.[19] The overall stoichiometric equations were created by adding up for all electron acceptors and donors based on the e^- eq fractions in Tables 2 and 3. Table 4 summarizes the stoichiometric reactions of biohydrogen production as a function of initial glucose concentration and pretreatment agent.

As shown in Table 4, the yield of biohydrogen production was decreased with increasing influent glucose concentration for both pretreatment. In case of NaOH pretreatment, as the glucose concentration increased from 3.75 to 15 g/L, the biohydrogen yield decreased from 2.49 to 1.63 mol H_2/mol glucose. Furthermore, when KOH was used as a pretreatment agent, the biohydrogen yields were inversely associated with glucose concentration. Van Ginkel *et al*. operated two identical fermentors at different feed concentrations and reported that with decreasing the glucose loading rate from 18.9 to 0.5 g glucose/h, the yield of hydrogen production improved from 1.7 to 2.8 mol H_2/mol glucose.[20] In contrast, Shida *et al*. reported that as the organic loading increased from 19 to 140.6 g glucose/L/day, the biohydrogen production increased from 12 to 76 L/day.[27]

Overall, the acetate-butyrate pathway fermentation showed higher biohydrogen yield than acetate-ethanol pathway. When KOH was used as a pretreatment agent, the biohydrogen yields were 2.12, 1.96, 1.5, and 1.2 mol H_2/mol glucose for 3.75, 7.5, 11.25, and 15 g/L of influent glucose, respectively. Compared with the KOH, the yield of biohydrogen was enhanced by 17.5%, 7.1%, 18.7%, and 35.8% for 3.75, 7.5, 11.25, and 15 g/L of influent glucose, respectively, during NaOH pretreatment (acetate-butyrate pathway).

Based on the energy conservation aspect, the acetate formation is the favor pathway for fermentor microorganisms during production from acetyl-CoA and also has led to reduced soluble end product or H_2. This situation attributed to typical mesophilic fermentation types including acetate-butyrate, acetate-ethanol, and acetate-propionate.[17] *Clostridium pasteurianum* is used with acetate-butyrate pathway for producing hydrogen and the highest obtained biohydrogen yield by these bacteria is 4 mol H_2/mol glucose.[20,23] Conversely, the theoretical yield of hydrogen according to acetate-ethanol pathway is 2 mol hydrogen/mol glucose.[12,22] The synthesis of ethanol from acetyl-CoA can produce reduced ferredoxin and also H_2 generation. The lower biohydrogen production during KOH pretreatment presumably related to electron flows from reduced ferredoxin to NAD^+ and produced $NADH_2$ instead of biohydrogen.[18,21] The reduction of acetyl-CoA can produce ethanol and butyrate and lead to $NADH_2$ consumption and may result in lower H_2 production.[18]

Conclusions

This study evaluates the biohydrogen production and stoichiometry reaction of glucose fermentation by acetate-butyrate and acetate-ethanol pathway induced by sludge pretreatment. Based on the obtained results, the following conclusions can be drawn:

- The percentage of hydrogen in the biogas produced ranged from 40% to 44%
- The highest biohydrogen production was obtained at 15 g/L of influent concentration with respect to 2.6 ± 0.5 L and 2.2 ± 0.7 L for NaOH and KOH pretreatment, respectively
- The glucose conversion efficiency during batch test was more than 90%
- For all tests, the mass balance recoveries based on the e^- eq were closed within 5.2%–13.5%
- The acetate-butyrate pathway fermentation showed higher biohydrogen yield than acetate-ethanol pathway.

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None.

Conflicts of interest

There are no conflicts of interest.

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