

# The Effect of Natural Buffer on Biohydrogen Production

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**Abstract:** Hydrogen is the promising ideal energy carrier with no emission but water on its combustible in the next generation. Hydrogen production using biological methods is greener than other methods using fossil fuel. One of the major factors affecting the operation of biohydrogen production is pH level in bioreactors. Restrain of declining pH is expected to increase hydrogen production. Pretreatment is one key factor in successful biohydrogen fermentation using mixed microbes. This study aims to investigate the natural buffer effect on biohydrogen using hot compressed water pretreatment. This batch fermentation experiment was operated in a 110 mL glass reactor with 3.75 g/L glucose as substrate. Mixed culture was obtained from cow dung compost treated with hot compressed water pretreatment at 150 °C, 0.5 MPa for 40 minutes. Fine dried eggshell powder and calcinated eggshell were added with 1 g/L, 3 g/L, and 5 g/L concentrations as buffer agents. The result showed that the addition of 1 g/L eggshell obtained the highest hydrogen production rate of 0.92 mol H<sub>2</sub>/mol glucose. Butyric acid and acetic acid are recognized as an indicator of hydrogen production and the Butyric/Acetic molar ratio over 2.6 as efficient biohydrogen fermentation. The highest B/A ratio in this experiment was 4.62 on 3g/L addition of eggshell powder.

**Keywords:** Hot compressed water, biohydrogen, eggshell waste, calcinated eggshell.

## Introduction

Anaerobic fermentation is performed under fermentation conditions with limited or discharged oxygen. It is an alternative to decompose complex waste material with some benefits. Energy generation, waste reduction, and valuable end-product are commonly promising advantages [1,2]. Biological hydrogen is one of the energy sources generated from anaerobic fermentation. There are two types of fermentation that produce bio-hydrogen: with the presence of light or photo fermentation and without light or dark fermentation. Dark fermentation is preferable for practical applications because of its cost, energy-saving, and broad feedstock [3].

There is a wide range of substrate resources for biohydrogen production. Many experiments were conducted using wasted biomass with many researchers. For instance, fruit peel, beer lees, peach pulp [4], tofu processing wastewater and residue [5, 6, 7] and other biomass resources [8, 9, 10, 11, 12, 13]. Every biomass contains specific nutrition for supporting bacteria growth in biohydrogen production. Separately, [14] declare that high hydrogen yield can be achieved by adjusting the C/N ratio in optimum conditions.

Microbe sources, pretreatments, substrates, and other factors may influence dark fermentation or biohydrogen fermentation. Pretreatment can be applied for both microbes and feedstock. Generally, there are four categories of pretreatment methods: physical, chemical, biological, and physicochemical. At physical/mechanical pretreatment, the size reducing technique is commonly used for lignocellulosic biomass [15]. Chemical and physicochemical treatments are frequently adopted because of the process feasibility. In biohydrogen fermentation, a carbohydrate, such as a monosaccharide or a disaccharide, has high biodegradability. Hence, a number of experiments use these carbohydrates as a carbon source [16].

Inoculum is very important to start biohydrogen fermentation. Both pure culture and mixed culture are applied in biohydrogen fermentation. Many studies achieved high hydrogen yield while using pure cultures, such as *Enterobacter sp.*, *Clostridium sp.*, *Caldicellulosiruptor saccharolyticus*, or *Thermotoga neapolitana* [17, 18, 19, 20, 21]. The usage of mixed culture has more technical and economic advantages than pure culture [22].

High temperature (heat pretreatment) is mostly used to suppress methanogenic bacteria and enrich hydrogen-producing bacteria in biohydrogen fermentation. Recently, the hot compressed water treatment was developed as a bacteria screening method. This technique can promote *Clostridium sp.* to produce hydrogen more than heat treatment [23]. Both

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inoculum and substrate interaction, as an inoculum-substrate ratio, influence the production of biohydrogen [24].

In addition to biohydrogen gas, organic acid is also produced in the reactor. Acetic, butyric, and propionic acid are mainly produced during hydrogen fermentation. The case of [25] reported that acetic and butyric acid correlates with carbohydrate and protein from substrate added during biohydrogen production. Therefore, these organic acids will accumulate in the reactor, drop the pH value, and subsequently interrupt hydrogen production. Other fermentation types also produce ethanol as a by-product. Therefore, the effect of acid on hydrogen inhibitory is higher than that of ethanol [26]. Furthermore, the accumulation of lactic acid, which produces low substrate concentration, can also inhibit biohydrogen production [27]. Hence, controlling pH value in the reactor is one of the critical factors to successful biological hydrogen production.

The influence of pH in dark biohydrogen production is mainly related to the feedstock's metabolism used during the fermentation period. Hydrogenase is an enzyme that plays a role in pH changes during hydrogen production. Generally, a more acidic pH value is always considered favorable. It was reported that the best pH for fermentation should be in the range of 5.5 to 6.0. The highest yield of hydrogen using mixed microflora as a culture was obtained at a pH of 4.5. It is also observed that the changes of pH are attached to the bacterial action on the substrate during production. Hence, the most important is obtaining the first initial value of pH and managing the value over the period of production [28]. To keep the performance of an acidogenic digester stable, it is necessary to add a pH buffer to neutralize the increasing VFAs and counteract the pH decrease. In previous studies, sodium carbonate and bicarbonate ( $\text{Na}_2\text{CO}_3$  and  $\text{NaHCO}_3$ ), calcium carbonate ( $\text{CaCO}_3$ ), and ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ) were used as pH buffers for anaerobic fermentation [29].

**Table 1.** Culture solution composition

Mineral	Unit	Amount
$\text{NH}_4\text{Cl}$	g/L	1.33
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	g/L	0.1
$\text{NaCl}$	mg/L	10
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	mg/L	10
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	mg/L	10
$\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$	mg/L	13
$\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$	mg/L	4.37
$\text{KH}_2\text{PO}_4$	g/L	5.99
$\text{K}_2\text{HPO}_4$	g/L	1.05
Distilled water	mL	until 1000

Eggshells can be alternatively used as a natural source of calcium and are characterized by high concentrations. The eggshell consists of 95% of calcium carbonate, 3.5% of glycoproteins, and proteoglycans. In addition, the inner shell membrane contains glucosamine, chondroitin sulfate, hyaluronic acid, type I collagen, and a high amount of proteins and microelements such as magnesium, strontium, zinc, barium, fluorine [30].

This study applied wasted biomass (eggshell) as a pH buffer to prevent a sharp drop in pH value. The eggshell contains around 95 % calcium carbonate [30]. This chemical substance is commonly known as

a buffering agent. The case provided by [31] uses a very high concentration (64 g/L) of chicken eggshells as a buffer for hydrogen production. A high concentration of  $\text{CaCO}_3$  interferes with hydrogen fermentation [32]. Therefore, low chicken eggshell powder concentration will be used in this study. There is limited information about the calcined eggshell effect on biohydrogen production. Therefore, eggshell and calcined eggshell utilization on biohydrogen production become a focus of this study. Preventing a sharp drop in pH value is expected to continue fermentation and increase hydrogen production. In the previous study, anaerobic digestion sludge was used as a bacteria source. The HCW is not adapted to another bacterial source yet. Hence in this study, we explore another bacteria source as an inoculum.

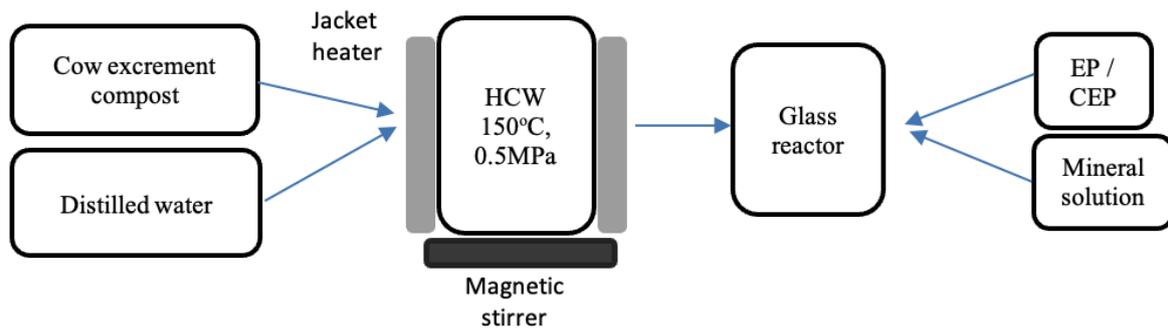
## Methods

### Inoculum and substrate source

In this study, a pretreated mixed culture is used to produce hydrogen. Mixed culture was obtained from cow excrement compost. Compost was dried by air in a shaded place for three days. Dried compost was comminuted and sieved for 0.5 mm to store at room temperature before use. Hot compressed water pretreatment was applied to screen hydrogen-producing bacteria.

The substrate of 3.75 g/L glucose was used to support bacteria growth. Micronutrient in the form of mineral culture solution contains  $\text{NH}_4\text{Cl}$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{NaCl}$ ,  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ . The amounts of each mineral are as shown in Table 1.

A 300 mL pressure resistance tube was used to perform HCW pretreatment. A pressure gauge and a temperature sensor were attached to monitor the condition inside the tube. This tube had two valves. The first valve for pressure elevation is attached to the nitrogen tank. Another valve was used to release gas and reduce the pressure inside the tube. An outer



**Figure 1.** Schematic diagram of experiment procedure

electric mantel heater was used to elevate temperature.

### Pretreatment

**Inoculum.** Dried fine compost was mixed with distilled water (ratio 1:10 w/v) then put into a pressure resistance tube of 300 ml for hot compressed water (HCW) pretreatment. The condition of this pretreatment was set at 150 °C temperature, 0.5 MPa pressure, and 40 minutes holding time. This condition is proven to suppressed methanogens bacteria [23]. Nitrogen gas was filled into the headspace of the HCW device to create a pressurized condition, and an automatic electric jacket heater was covered to increase temperature. This pretreatment method was adapted from [33], which is similar to NED (Nitrogen Explosive Decompression) method by [34]. After the HCW pretreatment, the inoculum is ready to use.

**Natural Buffer.** Waste eggshell was used as a natural pH buffer. Two treatments were applied to waste eggshells: eggshell powder (EP) and calcinated eggshell powder (CEP). EP was made of drying eggshell at 110 °C for 24 hours, then ground and sieved at 0.5 mm. CEP was made by heating EP at 900 °C for 2 hours. Concentration conditions of EP and CEP added to fermentation reactor were varied at 1 g/L (EP1 and CEP1), 3 g/L (EP3 and CEP3) and 5 g/L (EP5 and CEP5). Treatment without the addition of EP or CEP was defined as Control (C) treatment.

### Anaerobic fermentation

Triplicate batch fermentation ran in batch mode using a 125 mL glass reactor with 110 mL working volume. Each reactor contained 100 ml mineral culture solution, 10 ml pretreated inoculum, and 3.75 g/L glucose as a substrate. The reactor was closed with a rubber stopper then sealed with an aluminum cap. Nitrogen gas was sparged into the reactor's headspace for 1 minute to present an anaerobic environment.

All reactors were put into a shaker incubator (Eyela, MMS-310, and Eyela, LTI-601SD) at a shaking rate of 70 rpm and mesophilic temperature of 37 °C. The generated gas was collected and measured with a syringe every 12 hours. Gases were calculated and converted into standard conditions (STP, Standard Temperature, and Pressure). A gas chromatograph measured all gases with a thermal conductivity detector (Shimadzu, GC TCD 14B with nitrogen gas carrier) using the Porapack Q column (Agilent Tech.) to determine composition concentrations. The minimum gas volume using GC was 1 mL, which caused the case of gas generated less than 1 mL to be injected into GC. Hydrogen fermentation runs for 156 hours. The pH value in reactors was not controlled or adjusted during the experiment.

At the end of fermentation, pH, the concentration of glucose and organic acid were measured from fermentation liquid using pH meter (Toadkk, HM-21P), HPLC (for organic acid) attached with Shim-pack SCR-102H column and CDD-6A detector, HPLC (for sugar) with Shim-pack ISA07/S2504 column and RF-10AXL detector (Shimadzu, Prominence).

### Data analysis

The modified Gompertz model is a common model that is widely used to express hydrogen production [35]. The mean experimental data were fitted with the modified Gompertz equation (Equation (1)) to determine  $H_p$ : Hydrogen potential (mL);  $r$ : hydrogen production rate (mL/h);  $l$ : lag time (h); and  $t$ : time (h) [36].

$$H(t) = H_p \exp \left\{ \exp \left[ \frac{r}{H_p} (\lambda - t) + 1 \right] \right\} \quad (1)$$

After 3 parameters obtained ( $H_p$ ,  $r$  and  $\lambda$ ), the eq. (1) was used to determine hydrogen yield at 95 percent, namely  $t_{95}$ . This equation (eq. 2) was derived from the modified Gompertz eq. (1). The eq. (2) was defined by [37] and applied to compare and evaluate the experimental condition [38, 39].

$$t_{95} = \frac{H_p}{r_e} (1 - \ln(-\ln 0.95)) + \lambda \quad (1)$$

$$r_{H_2} = \frac{H_{max}}{\lambda + H_{max}/r_{max}} \quad (2)$$

Another experimental condition that derived from Gompertz equation was the average of hydrogen production rate ( $r_{H_2}$ ). The  $r_{H_2}$  was calculated based on Equation (3) [40]. Solver add-in function from Microsoft Excel 365 was used to minimize the sum square error between experimental data and modified Gompertz model. MS. Excel was also used to determine the coefficient correlation ( $R^2$ ) among them.

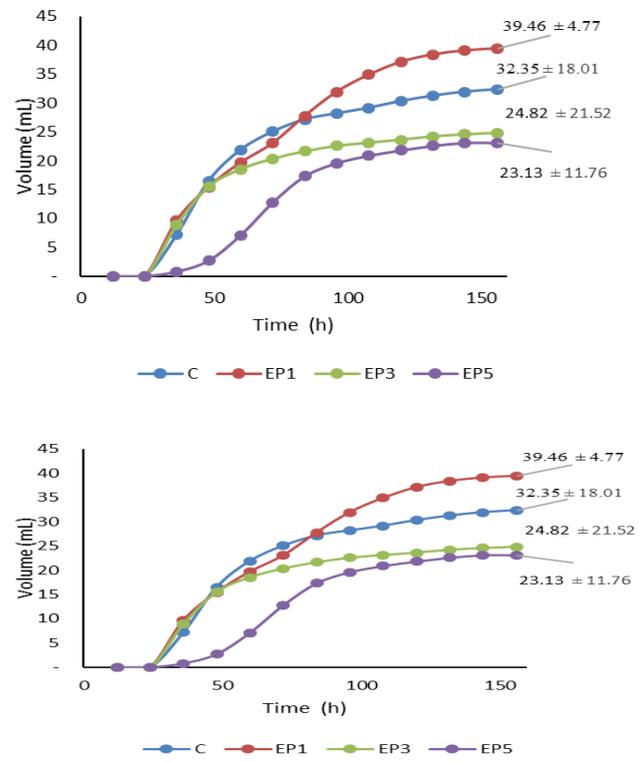
## Results and Discussions

### Gas Generation

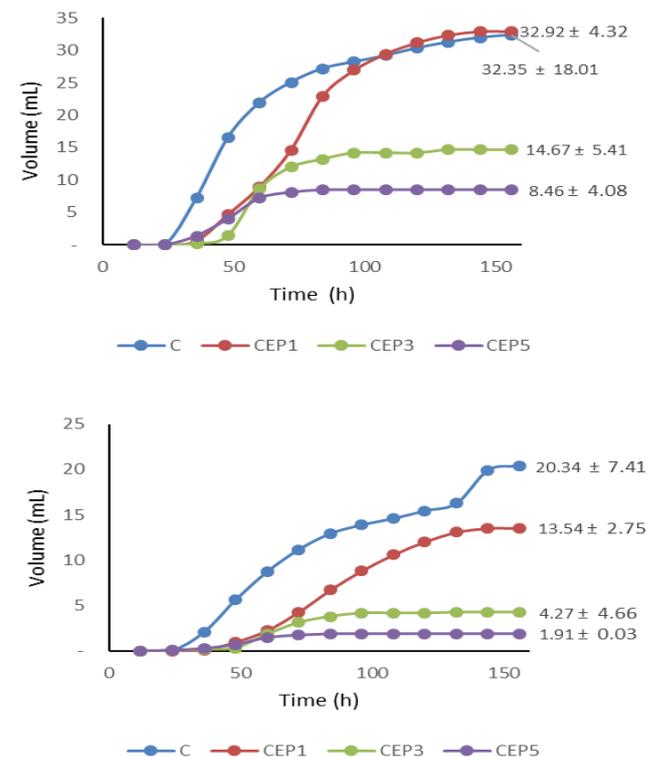
Gas productions of each treatment section are shown in Figure 2 and Figure 3. A small amount of gas was produced during the first 24 hours, but less hydrogen or carbon dioxide was detected. After 24 hours, hydrogen ( $H_2$ ) and carbon dioxide ( $CO_2$ ) are generated during fermentation. There was no methane gas produced during biohydrogen fermentation. It is shown that HCW pretreatment on excrement compost successfully suppresses methanogens bacteria. The experimental  $H_2$ - $CO_2$  production is shown in Figure 2 and Figure 3. The maximum hydrogen concentration on the sample was detected at 76 %. On adding one g/L eggshell powder (EP1), hydrogen production potential achieved in the highest yield was 0.92 mol  $H_2$  / mol glucose. It was higher compared with the control sample (0.66 mol  $H_2$  / mol glucose). Other treatments showed lower  $H_2$  gas production than EP1. EP3 and EP5 treatment produced hydrogen potential with a slightly different amount. It was 0.51 mol  $H_2$  / mol glucose for EP3 and 0.52 mol  $H_2$  / mol glucose for EP5, respectively. The control treatment achieved a hydrogen potential of 0.66 mol  $H_2$  / mol glucose (See Table 2). The hydrogen production fitted well to the modified Gompertz equation model with  $R^2 > 0.989$ . With the addition of a buffer (EP&CEP), bacteria's adaptation time (lag time) occurs variously. The addition of 1 and 3 g/L EP gained a shorter lag time when compared to the control sample. Otherwise, the addition of 5 g/L EP and CEP at all concentrations increased the lag time with the longest lag time, 46.60 hours.

Calcination was expected to enhance the alkalinity in the reactor. We added one g/L calcined eggshell (CEP1) generated hydrogen potential ( $H_p$ ) gas of 0.71 mol  $H_2$  / mol glucose. The hydrogen potential sharply dropped to 0.31 mol  $H_2$  / mol glucose and 0.18 mol  $H_2$  / mol glucose after putting 3 g/L and 5 g/L CEP, respectively. Both calcined eggshell and

eggshell powder increased the hydrogen yield at 1 g/L addition, but eggshell powder increased higher than calcined eggshell.



**Figure 2.** Gas production of C and EP treatment (a) hydrogen and (b) Carbon dioxide.



**Figure 3.** Gas production of C and CEP treatment (a) hydrogen and (b) Carbon dioxide.

**Table 2.** Hydrogen production parameters using modified Gompertz equation

Sample	Lag time (h)	$r_{max}$ (mL/h)	H Potential (mL)	R <sup>2</sup>	H <sub>2</sub> Yield (mol H <sub>2</sub> / mol glucose)
C	25.31	0.68	30.57	0.994	0.66
EP1	12.78	0.41	42.73	0.991	0.92
EP3	17.55	0.49	23.90	0.989	0.51
EP5	41.92	0.39	24.37	0.996	0.52
CEP1	43.52	0.58	33.33	0.998	0.71
CEP3	46.60	0.63	14.26	0.998	0.31
CEP5	31.32	0.26	8.59	0.997	0.18

**Table 3.** The experimental condition in the reactor

Sample	$t_{95}$ (h)	$r_{max}$ (mL/h)	$r$ average (mL/h)
C	90.8	0.68	0.44
EP1	163.2	0.41	0.37
EP3	88.5	0.49	0.36
EP5	132.8	0.39	0.23
CEP1	128.0	0.58	0.33
CEP3	79.5	0.63	0.21
CEP5	80.2	0.26	0.13

The hydrogen potential was declining along with the increase of buffer concentration. This is because the eggshell primarily contains CaCO<sub>3</sub> [30]. Even though the solubility of CaCO<sub>3</sub> was low in pure water, the addition of CaCO<sub>3</sub> in low concentration increased hydrogen production. But the presence of high CaCO<sub>3</sub> concentration (> 4 g/L) promotes lactate-type fermentation, which causes hydrogen fermentation to be halted [32].

Hydrogen yield production time ( $t_{95}$ ) changed in the addition of EP/CEP buffer (See Table 3). At sample EP1, the fermentation process still occurs after 156 hours. However, it showed that at 163 hours of fermentation time, 95 % of hydrogen potential was produced. The other samples showed variation time of hydrogen production. It might be due to buffer activity in the reactor. In the CaCO<sub>3</sub> buffer system, Ca<sup>2+</sup> will be released in acid conditions (acid production during hydrogen fermentation). The previous studies [41], [42] showed that the presence of calcium ions would influence biohydrogen production.

The production rate of biohydrogen changed after the addition of EP and CEP. The control sample achieves the highest  $r_{max}$  compared to all treatments. The  $r_{max}$  reached 0.68 mL/h with an average 0.44 mL/h. Among the treatment samples, the sample CEP3 gains the highest  $r_{max}$  with 0.63 mL/h. However, since it has a long lag time and short  $t_{95}$ , the average of  $r$  was low at 0.21 mL/h. The detail of  $r_{max}$  and average  $r$  of each treatment are shown in Table 3.

### Fermentation Liquid End-product.

The declining pH level during fermentation may influence hydrogen gas production. Organic acid

produced by bacteria reduced the pH level. Hence, the addition of EP was expected to refrain from the high decline of pH level. The initial pH of the samples was different due to the addition of EP and CEP. The control sample pH showed the largest pH declining; its lowest decrease was from 6.29 to 4.86. Hydrogen-producing bacteria, *Clostridium* strain, has various inhibitory pH values. pH 4.6–5 is known as inhibitory value for some strains [43].

A higher concentration of EP refrained pH level decreasing more than lower concentration. EP component consists chiefly of CaCO<sub>3</sub>. However, CaCO<sub>3</sub> in high concentration could also promote lactic acid bacteria activity. For example, adding 3%, CaCO<sub>3</sub> increased lactic acid production by *Lactococcus lactis* [44]. According to Le Chatelier principle [31], the CaCO<sub>3</sub> will prevent the pH drop in the buffer system reaction, as shown in equation 4.

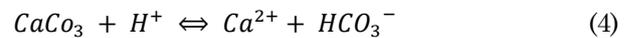


Table 4 shows the concentration of organic acids produced during fermentation after 156 hours. Butyric acid, lactic acid, and acetic acid are the primary organic acid generated during fermentation. Those organic acids are identified as the end-product of butyric-type fermentation by *Clostridium sp.* A high concentration of lactic acid shown in EP5 indicates that lactic acid-type fermentation occurred in this experiment. In CEP1 and CEP3 samples, no butyric acid was produced. It was expected that lactate-type fermentation occurs in the reactors. The absence of butyric acid in CEP1 and CEP3 leads to B/A molar ratio at 0.

The presence of butyric acid and acetic acid represented the effectiveness of biohydrogen fermentation because the butyrate/acetate (B/A) ratio could be a quantitative indicator of substrate metabolism and hydrogen production by anaerobic microflora. Furthermore, acetate is the precursor of two-thirds of methane production in meso- and thermophilic fermentation, the butyric type, and acetic type fermentation reaction, as shown in eq (5) and (6).



Butyric/Acetic (B/A) molar ratio higher than 2.6 indicates effective biohydrogen fermentation [19]. The highest B/A ratio achieved in this experiment was 4.62 (see Table 4). Lactate-type fermentation occurred on a higher concentration of EP and CEP treatments which was indicating by the lactic acid production.

**Table 4.** pH and main organic acid of liquid product

Treatment	Initial pH	Final pH	Butyric Acid (mg/mL)	Acetic Acid (mg/mL)	Lactic acid (mg/mL)	B/A molar ratio
C	6.29	4.86 - 5.08	452.0 - 1162.2	180.3 - 726.1	71.8 - 225.7	0.42 - 4.12
EP1	6.32	5.22 - 5.35	222.2 - 680.0	116.1 - 435.5	0.0 - 413.6	0.35 - 2.32
EP3	6.34	5.21 - 5.71	0.0 - 1430.0	106.0 - 244.7	0.0 - 1342.1	0.00 - 4.62
EP5	6.36	5.44 - 5.66	367.9 - 528.8	683.6 - 1839.2	1082.1 - 1767.1	0.20 - 0.44
CEP1	6.32	5.89 - 6.14	0.0	117.8 - 190.9	810.4 - 927.4	0.00
CEP3	6.38	6.35 - 6.37	0.0	113.3 - 855.8	671.6 - 982.9	0.00
CEP5	6.42	6.07 - 6.66	212.7 - 815.2	224.5 - 1989.3	530.8 - 1345.3	0.41 - 0.95

### Conclusion

The study has demonstrated that the addition of eggshell powder positively affects hydrogen fermentation, which is better than the calcined eggshell. This study's optimum eggshell powder addition was 1 g/L, resulting in an H<sub>2</sub> yield of 0.92 mol H<sub>2</sub> / mol glucose. CEP1 treatment reached a hydrogen yield of 0.71 mol H<sub>2</sub> / mol glucose, followed by Control and EP5 that were 0.66 and 0.52 mol H<sub>2</sub> / mol glucose, respectively. Butyrate-type fermentation occurred when a lower EP concentration was applied. With the addition of 1 g/L EP, the lag phase of bacterial growth decreases to almost half compared with the control sample.

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