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Optimization of Batch Dark Fermentation of *Chlorella* sp. Using Mixed-Cultures for Simultaneous Hydrogen and Butyric Acid Production

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Abstract: This paper reports on the optimum conditions for simultaneous hydrogen and butyric acid production from microalgae (*Chlorella* sp.) using enriched anaerobic mixed cultures as inoculum. The fermentation was objectively carried out under acidogenic conditions to achieve butyric acid for further ABE fermentation in solventogenesis stage. The main effects of initial pH (5 and 7), temperature (35 °C and 55 °C), and substrate concentration (40, 60, 80, and 100 g-VS/L) for hydrogen and butyric acid production were evaluated by using batch fermentation experiment. The major effects on hydrogen and butyric acid production are pH and temperature. The highest production of hydrogen and butyric acid was observed at pH 7 and temperature 35 °C. Using initial *Chlorella* sp. concentration of 80 g-VS/L or 100 g-VS/L at pH 7 and temperature 35 °C could produce hydrogen with an average yield of 22 mL-H_2/g-VS along with high butyric acid production yield of 0.05 g/g-VS, suggesting that microalgae (*Chlorella* sp.) has potential to be converted directly to butyric acid by using acidogenesis under above optimum conditions.

Keywords: microalgae; acidogenesis; hydrogen; butyric acid

1. Introduction

Currently, energy carriers or fuel derived from petroleum source have high requirements, while the petroleum reserve has been reduced [1]. The use of energy from the above energy sources is the cause of global warming. Nevertheless, demand for energy is likely to increase in the future, according to the forecast of Energy Information Administration 2016 (EIA2016) [2]. Therefore, alternatives such as hydrogen ethanol and butanol from biomass are used to resolve the petroleum-based energy crisis. Bio-hydrogen is a renewable energy that provides high energy value and does not cause greenhouse gas emissions during its combustion [3]. Bio-butanol as liquid fuel has high potential comparable to ethanol and gasoline and 96 octane number similar to gasoline. However, butanol has an energy content of 29.2 MJ/dm^3, which is higher than the ethanol energy content (19.6 MJ/dm^3) [4]. In addition,

the evaporation and corrosion levels of butanol are less than those of ethanol, thus it is possible for butanol to be directly used in gasoline engines without any engine modifications [5].

The Acetone-Butanol-Ethanol (ABE) fermentation process could potentially produce bio-butanol as the product from biomass, while hydrogen and carbon dioxide are simultaneously produced [6], with *Clostridium* sp. being the main bacterial group used for the production of butanol [7]. The ABE fermentation process has two distinct two stages. In the first acidogenasis stage, carbohydrates are majorly converted to butyric and acetic acids along with hydrogen and carbon dioxide. Subsequently, during the second solventogenesis stage, acetone butanol and ethanol are formed from butyric acid and acetic acid [7–9]. In addition, in general, when using mixed culture bacteria for fermentation, volatile fatty acids are produced during anaerobic dark fermentation [10,11] as a precursor for ABE fermentation. Therefore, volatile fatty acids can be produced with other types of microorganisms along with the butanol producing bacteria in the first stage dark fermentation process, in which mixed culture bacteria can be deployed instead [9,11–14].

The mixed-culture based bioprocess is better than pure bacteria, since organic biomass for biodegradation is a complex mixture in the form of carbohydrates (such as lignocellulosic hydrolysates), thus using mixed bacteria for the acidogenesis stage for anaerobic dark fermentation may be a superior alternative to the pure culture [15]. The specific advantage of mixed cultures over pure culture-based industrial biotechnology include no need for sterilization and can be adaptive due to microbial diversity, and the capacity to use mixed substrates [16]. Furthermore, strict and expensive sterilization of the fermenter and medium is not required for mixed-culture fermentation because the foreign bacteria cannot establish due to effective competition by members of the microbial community in the mixed culture [15]. Agler et al. [17] reviewed the capacity of a mixed-culture process for generating a mixture of short-chain carboxylates as an intermediate platform to generate complex fuels. Angennent et al. [10] and Agler et al. [17] reported that hydrogen partial pressure could thermodynamically direct fermentation pathway. When hydrogen partial pressure is less than 60 Pa, one mole of glucose would convert to two moles of acetic acid and four moles of hydrogen. In contrast, in a dark fermentation process at hydrogen partial pressure greater than 60 Pa, the direction of production is in the form of one mole of butyric acid and two moles of hydrogen. Thus, dark fermentation effluent normally contains mostly butyrate, which is favored for enhancing butanol production in the second stage of solventogenesis. Consequently, increasing butyric acid production during dark fermentation process can help to increase butanol production. Al-Shorgani et al. [18] reported that the butyric acid has an impact on butanol production, and butyric acid can increase butanol production with glucose addition to promoting bacteria metabolism.

Chlorella sp., *Scenedesmus* sp., *Chlorococum* sp., *Tetraselmis* sp. and *Chlamydomonas* sp. are unicellular microscopic algae that can grow easily in various water sources. They are mostly carbohydrates (starch and cellulose), the main substrate for biofuel production when using microbial fermentation, up to about 55% of dry weight [19]. Classified as the third-generation biomass for bio-fuel production, microalgae have advantages over the first-generation biomasses such as flour and sugar derived from food crops and the second-generation biomasses such as lignocelluloses in terms of biofuel production. Microalgae do not compete for arable land, can be grown in saline or freshwater environments, and can absorb CO_2. Furthermore, microalgae can be practically cultivated in open raceway ponds cost effectively [20]. Microalgae do not contain lignin and have fast growth potential [21,22]. Especially, *Chlorella* sp. are considered promising feedstock for ABE production because they have cellulose and hemicellulose of cell walls and accumulated starch as main carbohydrate sources (37–55% [23]). Most of the cell wall and starch can be converted to sugars for acid and ABE production [24]. The novelty of this research work was the investigation of the effects of three factors on the production of butyric acid and hydrogen during dark fermentation with enriched mixed cultures. In addition, we aimed to find the appropriate conditions for producing butyric acid and hydrogen from microalgae with mixed culture bacteria taken from the CSTR fermentation tank. Different sources of hydrogenic bacteria groups resulted in different productivities.

In this research, the major objective was to optimize conditions of mixed-culture dark fermentation for simultaneous hydrogen and butyric acid production from *Chlorella* sp. Effluents containing mainly with butyric were further used as inoculum for acetone-butanol-ethanol (ABE) fermentation. The main factors of initial pH (5 and 7), temperature (35 °C and 55 °C) and substrate concentration (40, 60, 80, and 100 g-VS/L) were tested under batch anaerobic dark fermentation.

2. Materials and Methods

2.1. Substrate and Inoculum Preparation

Microalgae (*Chlorella* sp.) purchased from Cheng Yang Instrument Corp, Taiwan as a dry, green powder. They were stored in a desiccator at room temperature prior to further use. The mixed cultures used as inoculum for fermentation were taken from APEC Research Center for Advanced Biohydrogen Technology (ACABT), Feng Chia University, Taichung, Taiwan. The inoculum was originally cultivated in the CSTR dark fermentation tank at the mesophilic temperature (35 °C) for hydrogen and acid production by feeding using sucrose at a concentration of 80 g-VS/L supplemented with Endo nutrients ($MnSO_4 \bullet H_2O$ 9.79 g/L, $FeSO_4 \bullet 7H_2O$ 25 g/L, $CuSO_4 \bullet 5H_2O$ 5 g/L and $CoCl_2 \bullet 6H_2O$ 0.125 g/L) [25]. The inoculum was later adapted to two different temperatures (mesophilic and thermophilic conditions), as have researchers demonstrated that mesophilic cultures can serve as sources for cultivation under thermophilic as well as hyperthermophilic conditions [26]. The obtained inoculum was acclimatized by adding 50 g-VS/L sucrose as a substrate without Endo nutrients and then leaving for three days at 150 rpm in an incubator shaker at mesophilic (35 °C) or thermophilic temperature (55 °C).

2.2. Bath Experiment for Acidogenesis Stage for Hydrogen and Butyric Acid Production

The batch experimental assay was first carried out to investigate the effect of either adding or not Endo nutrients on hydrogen production from *Chlorella* sp. in batch fermentation at the initial concentration of 80 g-VS/L and pH 7 with 15 mL total volume of serum bottle. The serum bottle contained 80 g-VS/L of *Chlorella* sp. and 4 mL of mixed cultures with or without Endo nutrient (the concentration of Endo nutrients: 9.79 g $MnSO_4 \bullet H_2O$, 25 g $FeSO_4 \bullet 7H_2O$, 5 g $CuSO_4 \bullet 5H_2O$ and 0.125 g $CoCl_2 \bullet 6H_2O$ in 1 L). The amount of nutrients used depended on the working volume (g-nutrient /L-working volume). The initial pH 7 ± 0.1 was adjusted with 0.5 M phosphate buffer (1.5 mL) followed by distilled water to 10 mL working volume. The serum bottles were capped with tight rubber stoppers and aluminum, and the headspace was flushed with 5 min of nitrogen gas to ensure anaerobic conditions. They were then placed in a batch incubator shaker at 150 rpm and 35 °C. Gas production from anaerobic dark fermentation was measured using a 50 mL glass syringe drawing from the headspace of the batch serum bottles for cumulative gas values and, for the composition gas hydrogen production, 1 mL plastic syringes were drawn daily. Both were analyzed with gas chromatography. For optimization of hydrogen and butyric acid production from *Chlorella* sp. using mixed cultures as a substrate, it was carried out at temperatures of 35 °C and 55 °C (two levels), the initial pHs of 5 and 7 (two levels) and the initial substrate concentrations of 40, 60, 80, and 100 g-VS/L (four levels) to determine the optimum conditions in batch dark fermentation of *Chlorella* sp. by using Taguchi method to aid experimental design.

The Taguchi method as a statistical tool for biotechnological applications is an easy and popular method used for experimental design. As reviewed by Rao et al. [27], comparative studies between response surface methodology (RSM) and the Taguchi techniques revealed that both techniques have similar results, however Taguchi technique requires half the time as RSM technique. Thus, Taguchi technique was selected for this investigation. Table 1 shows a set of experimental assays created using Taguchi design method. Eight batch test sets were used for the Taguchi design. However, in the experiment, there was one additional set of experiments (Batch Set 7), with the intensity of the substrate with 80 g-VS/L, at pH 7 and temperature 35 °C to compare the same initial substrate concentration and temperature with different pH. Thus, nine total batch test sets were performed. The batch fermentation

was conducted in 235 mL serum bottles by adding 60 mL of enriched inoculum of the mixed culture. The substrate was added at different initial concentrations of *Chlorella* sp. (40, 60, 80 and 100 g-VS/L) with 63 mL distilled water. Sucrose was used as positive control assay, representing microalgae and the blank assay was added with only distilled water, i.e., no substrate. 3N Hydrochloric acid and 3N sodium hydroxide were added to adjust to the initial of pH 5 ± 0.1 and 7 ± 0.1, respectively. Then, 22 mL of 0.5 M phosphate buffer was used in each bottle, followed by distilled water to 150 mL working volume. The bottles and microalgae were not sterilized before use and the fermentation was followed above. The fermentation broth was taken from each bottle after batch fermentation finished to measure pH and volatile fatty acids (VFAs).

Table 1. Experiment from apply of Taguchi design.

Batch Run	Conc. (g-VS/L) A	pH B	Temp. (°C) C
1	40	5	35
2	60	5	35
3	80	7	35
4	100	7	35
5	40	7	55
6	60	7	55
7	80	7	55
8	80	5	55
9	100	5	55

2.3. Analysis Methods

Total solid (TS), volatile solid (VS), ash, total Kjeldahl nitrogen (TKN), chemical oxygen demand (COD), and oil and grease were determined in accordance with standard methods described by APHA 1998 [28]. Total sugar was analyzed by Anthrone sulfuric acid method [29]. Components of microalgae were analyzed using Ion Chromatography Plasma (ICP) (main minerals analyzed: Na, K, Fe, Mg, Ca, As, Cr, Cd, Cu, Pb, Zn, and Mn). The suspensions were centrifuged at 10,000 rpm for 10 min with control temperature of 4 °C and the supernatants were filtered through nylon membrane with hole size of 0.45 μm into 2 mL vial bottle. Volatile fatty acids (VFAs) including acetic acid, propionic acid and butyric acid were analyzed using high performance liquid chromatography equipped with SphereClone^{TM} 5 μm ODS (2) 80 Å, LC Column 50 × 4.6 mm, Ea, with stationary phase: C18, UV-detector WL 210 nm Hitachi L7400. The operating temperature was 25 °C with Solution A 90% of 0.5 mM H_2SO_4 and Solution B 10% of 99.9% methanol as mobile phase at a flow rate of 0.6 mL/min, and the injection sample volume was 20 μL. The concentration of acetic acid, propionic acid and butyric acid were calculated using the linear equation obtained from various concentrations (0.05, 0.1, 1, 5 and 10 g/L) of standard mixed acid solutions. The concentration of hydrogen gas, nitrogen gas and carbon dioxide gas were analyzed by gas chromatography (GC) equipped with thermal conductivity detector (China Chromatograph Personal GC 1000). Argon gas was used as a carrier gas flow into 1/8 mm ID × 4 m Steel column (Porapak Q 10%). The temperatures of injection, oven and detector were 40, 28, and 40 °C, respectively.

The cumulative hydrogen was calculated from the linear equation of standard gas (hydrogen gas, nitrogen gas, and carbon dioxide). Then, the gas concentration obtained was used to calculate the hydrogen volume from total gas. The hydrogen gas on each day was combined to calculate the cumulative hydrogen (unit: mL-H_2). Hydrogen production yield was calculated from cumulative hydrogen by dividing it by the volatile solid of initial substrate concentration (unit: mL-H_2/g-VS).

3. Results

3.1. Chlorella sp. Characterization and Batch Fermentation of Chlorella sp. with and without Endo Nutrients

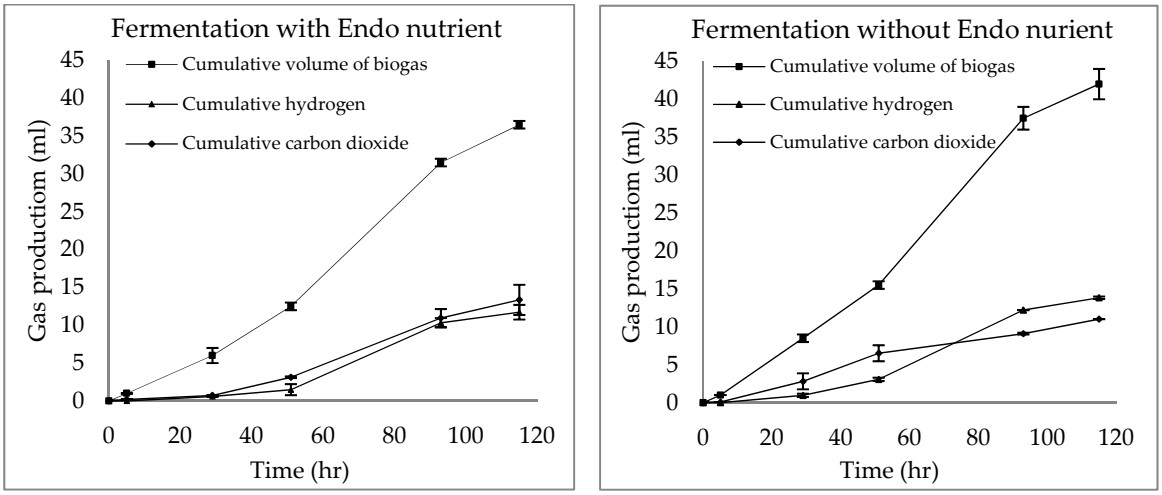
The characterization of *Chlorella* sp. is shown in Table 2. TS of 97.7% consisted of VS of 91% and ash of 6.7% (w/w). TKN, total sugar, oil and grease of 4.6%, 6.1%, and 2.6% (w/w), respectively, were recorded. *Chlorella* sp. microalgae in powder form had high volatile solids and low ash, indicating high organic matter suitable for biodegradation [30,31]. Essential elements for bacteria growth such as Mg, Fe, Cu, K, and Co [32,33] was also found in *Chlorella* sp. Although Co was not analyzed in this study, it was previously found as one of the main elements in *Chlorella* sp. [34].

Table 2. Characterizations of the *Chlorella* sp.

Character of *Chlorella* sp.	Value	Character of *Chlorella* sp.	Value
TS % (w/w)	97.7	Mg (mg/kg)	2458.66
VS % (w/w TS)	91.0	Ca (mg/kg)	1919.06
Ash % (w/w TS)	6.7	As (mg/kg)	ND
Total Sugar % (w/w)	6.1	Cr (mg/kg)	5.22
COD (g-COD/g-VS)	1.43	Cd (mg/kg)	ND
Oil and grease % (w/w)	2.6	Cu (mg/kg)	7.4
TKN % (w/w)	4.6	Pb (mg/kg)	5.22
Na (mg/kg)	248.04	Zn (mg/kg)	110.1
K (mg/kg)	7267.19	Mn (mg/kg)	48.74
Fe (mg/kg)	574.41		

ND, not detected.

Cumulative hydrogen generated from batch fermentation with and without Endo nutrients was 12 ± 1.0 and 14 ± 0.2 mL-H_2, respectively, under mesophilic temperature (35 °C) and initial pH 7. The compositions of gas production in this study is shown in Figure 1. Hydrogen represented around 46% of total gas generated from batch without Endo nutrients. Meanwhile, the batch fermentation with Endo nutrient had hydrogen content of 40% of total gas generated, i.e., slightly lower than that from the batch fermentation adding Endo nutrient, demonstrating that *Chlorella* sp. itself contained sufficient nutrients for bacteria growth [35]. In the fermentation process, microorganisms degrade complex biomass prior to taking up nutrients and trace elements for microbial metabolism activities [36]. The hydrogen production yields of anaerobic fermentation from *Chlorella* sp. with and without nutrient were 14.66 ± 1.2 and 17.29 ± 0.2 mL-H_2/g-VS, respectively. The effect of nutrients ($FeSO_4 \bullet 7H_2O$, Urea, and Na_2HPO_4) was previously studied by Yossan et al. [13]. They found $FeSO_4 \bullet 7H_2O$ Urea and Na_2HPO_4 were important nutrients affecting hydrogen production at a proper amount, which were sufficiently contained in the substrate used. Batch dark fermentation of *Chlorella* sp. with Endo nutrients provided lower hydrogen yield than batch fermentation without Endo nutrients, indicating too high amount of important minerals achieved by adding Endo nutrients could be toxic instead of enhancing the microorganisms. Hydrogen yield obtained from the batch fermentation without adding nutrient is in accordance with hydrogen yield result reported by Wieczorek et al. [34]. The batch dark fermentation of *Chlorella vulgaris* at different initial concentrations of 5, 10, 20 and 30 g-VS/L at 60 °C without adding nutrients could provide highest hydrogen yield of 19 mL-H_2/g-VS at initial concentration of 10 g-VS/L. Therefore, the fermentation of hydrogen production from *Chlorella* sp. without Endo nutrient was selected to further optimize the conditions of pH, temperature and initial concentration for batch anaerobic dark fermentation.



(A) (B)

Figure 1. Cumulative gas production (hydrogen and carbon dioxide) from *Chlorella* sp. in batch fermentation with **(A)** and without **(B)** Endo nutrient.

3.2. Hydrogen Production

In dark fermentative system, hydrogen and carbon dioxide are simultaneously generated with volatile fatty acids (VFAs). Butyric acid, a major VFAs generated from dark fermentation, can be converted to butanol by pure culture-based ABE fermentation [37]. In this research, *Chlorella* sp. was used as the main carbon source under various conditions during batch fermentation using mixed acidogenic bacteria, resulting in different amounts of gases. Figure 2 shows the cumulative total gas and hydrogen production as well as the concentration of hydrogen in the bio syngas for the nine batch sets using *Chlorella* sp. as a substrate and sucrose for control batch fermentation set. The different types of substrate affected the batch anaerobic dark fermentation [38]. Only Batch Sets 3 and 4 of sucrose fermentation could produce hydrogen of 199 ± 17 mL-H_2 (17 mL-H_2/g-VS) and 170 ± 14 mL-H_2 (10 mL-H_2/g-VS), respectively, which were lower than Batch Sets 3 and 4 of *Chlorella* sp. fermentation. As synthetic nutrients were not added for this optimization, higher hydrogen production obtained from *Chlorella* sp. fermentation could be strong evident that nutrients contained in *Chlorella* sp. could enhance hydrogen production. Therefore, not adding synthetic nutrients to the sucrose fermentation led improper nutrient concentrations for bacteria growth metabolism. Nutrients are necessary for enzymatic activities and cell growth in anaerobic digestion process [36]. Although nutrients were not adequate, batch sucrose fermentation under condition of 35 °C and initial pH 7 might be suitable for cell growth of microorganisms for hydrogen production [39]. The low initial pH 5 inhibited hydrogen production [40]. The initial concentration of substrate is an important parameter affecting microbial fermentation process [41]. The initial concentrations used in this study were 40, 60, 80, and 100 g-VS/L and did not differ significantly ($P_{value} > 0.05$) for hydrogen production under initial pH 7 and operating temperature of 35 °C or 55 °C. Cumulative gas production from *Chlorella* sp. was highest under Batch Set 4 (initial concentration 100 g-VS/L, pH 7 and 35 °C) with total cumulative gas of 646 ± 48 mL and cumulative hydrogen of 330 ± 32 mL-H_2. The productions of cumulative gas and cumulative hydrogen at Batch Set 3 were 592 ± 42 mL and 266 ± 25 mL-H_2, respectively, obtained under conditions of initial concentration 80 g-VS/L, pH 7 and 35 °C were insignificantly lower than those of Batch Set 4 ($P_{value} > 0.05$). However, higher initial concentration of substrate was more likely to result in more hydrogen production. Yun et al. [39] reported the optimum conditions of initial pH 7.4 at 35 °C with high initial concentration of *Chlorella vulgaris* 76 g/L could produce hydrogen yield of 31.2 mL-H_2/g of dry cell weight. Meanwhile, under thermophilic temperature (55 °C) and initial pH 7, the cumulative gas production (hydrogen production) obtained from *Chlorella* sp. at different initial concentration of

40, 60, and 80 g-VS/L (Batch Sets 5–7) were 349 ± 17 mL (107 ± 10 mL-H_2), 347 ± 19 mL (141 ± 13 mL-H_2) and 352 ± 21 mL (118 ± 12 mL-H_2), respectively. At the lower substrate concentrations, anaerobic dark fermentation caused a lower hydrogen productivity [42]. Increasing the initial concentration to a certain level can increase the hydrogen productivity [39]. Because the excess substrate concentration caused accumulation of cell and VFAs concentration, low pH could inhibit acidogenic bacteria in the fermentation process [39].

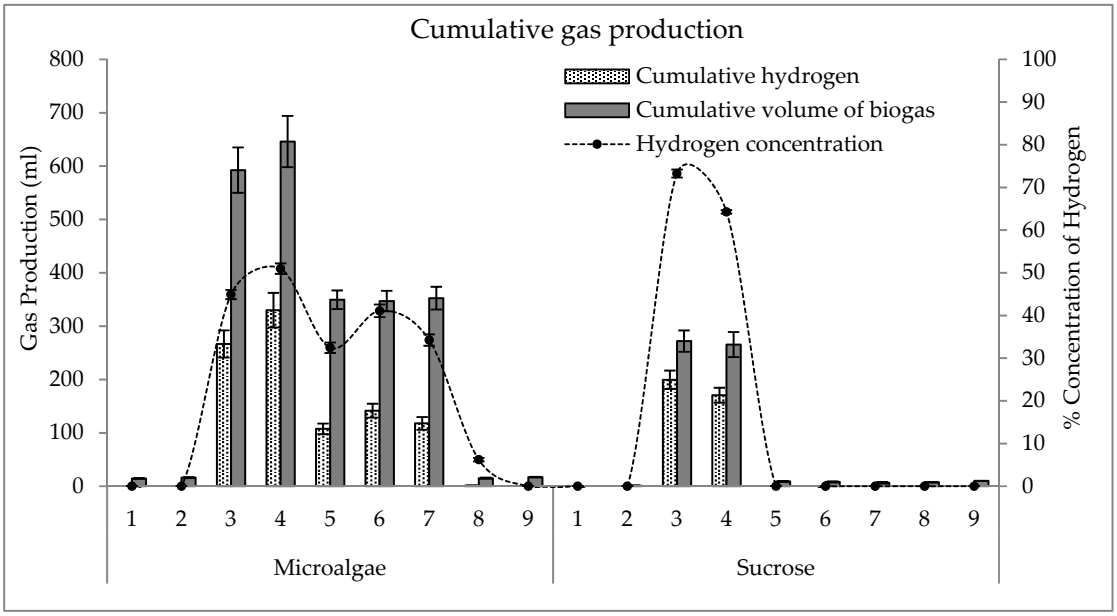


Figure 2. Hydrogen production of batch fermentation from microalgae (*Chlorella* sp.) with sucrose as a control set under different conditions for initial concentration, pH, and temperature (Batch Sets 1–9) based on Taguchi design.

Two operating temperatures, mesophilic temperature (35 °C) and thermophilic temperature (55 °C), were employed for fermentative hydrogen production under acidogenic anaerobic dark fermentation in batch mode. Temperature is an essential factor for microbial biodegradation to produce hydrogen and VFAs [43,44]. As demonstrated in Figure 3, at initial pH 7, hydrogen production yields obtained from *Chlorella* sp. fermentation at initial concentrations of 80 g-VS/L (Batch assay No. 3) and 100 g-VS/L (Batch assay No. 4) at 35 °C were 22.2 ± 2.1 mL-H_2/g-VS and 22.0 ± 2.2 mL-H_2/g-VS, which are higher than these obtained at 55 °C of 18.9 ± 1.6 mL-H_2/g-VS (initial concentration: of 40 g-VS/L of Batch Set 5), 15.9 ± 1.4 mL-H_2/g-VS (60 g-VS/L of run 6) and 10.1 ± 1.0 mL-H_2/g-VS (initial concentration: 80 g-VS/L of Batch Set 7). Those results demonstrate that the hydrogen production yields obtained from fermentation conducted at mesophilic (35 °C) conditions were higher than those at thermophilic (55 °C) conditions. Although the inoculum was brought from the mesophilic condition, in a mixed culture, some bacteria can grow at high temperatures, in accordance with the research of Stein et al. [12] and De la Rubia et al. [26] who used inoculum from a mesophilic condition for fermentation at thermophilic or even hyperthermophilic temperature. However, Qui et al. [43] studied the effect of temperature, finding that the mesophilic range of 35-40 °C yielded higher hydrogen production. They used active sludge from sewage treatment plant and the mesophilic range was familiar for microbes. In addition, the mesophilic condition (35 °C) was used by Yun et al. [39] for fermentation with *Chlorella vulgaris* at 31.2 mL-H_2/g of dry cell weight. Yokoyama et al. [45] presented lower hydrogen production at the temperature of 55 °C than at 35 °C, and the highest hydrogen production yield (392 mL-H_2/L of slurry) at 60 °C using cow waste slurry with anaerobic microflora. Thus, the production of hydrogen depends on the microbes used as inoculum. In our research, as the anaerobic sludge used as inoculum was taken from mesophilic operation conditions, operating at higher thermophilic temperature could lead

to some of the microorganisms being inhibited, resulting in decreased microbial community diversity and consequently reduced hydrogen productivity [43,45].

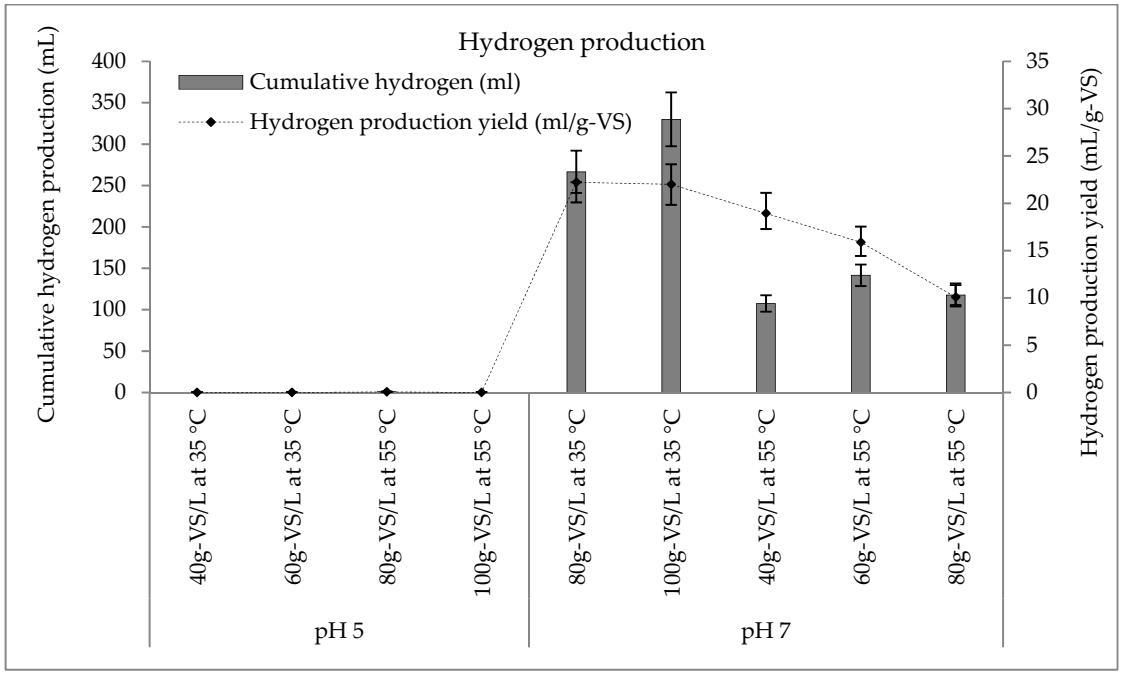


Figure 3. Cumulative hydrogen and hydrogen production yields from initial pH of 7 and 5 at different initial concentrations (40–100 g-VS/L) and temperature (35 and 55 °C).

The initial pH is another crucial parameter affecting fermentation systems for hydrogen production. It can affect the metabolism in anaerobic acidogenic fermentation pathway of microorganisms [46,47]. The effect of initial of pH 5 and 7 on hydrogen production yield of *Chorella* sp. was tests in various conditions, as shown in Figure 3. The hydrogen production yield obtained when using pH 7 was obviously different from when using pH 5. Hydrogen production from *Chlorella* sp. was successfully obtained by fermentation at pH 7 only for both temperatures and all initial substrate concentrations. Yun et al. [39] obtained the maximum hydrogen production of 30.74 mL-H_2/g-dry cell at pH 7 and 35 °C. In addition, the optimum conditions in several studies on anaerobic digestion were different for mixed culture including the pH range. For example, Yun et al. [39] found the optimum condition to be pH 7.4 (after testing pH 4.2–9) at 35 °C, while the optimum condition was pH 6 (after testing pH range 5.5–7.7) at 39 °C in the study by De Gioannis et al. [48]. Therefore, the optimal pH for dark fermentation depends on inoculum sources, enrichment of inoculum and type of substrate [36,49]. However, using initial pH 5 in this research could not produce hydrogen at either 35 or 55 °C in mixed-culture fermentation. Although the research of Fang and Liu [50] was studied the pH in the range of 4–7 to produce hydrogen from glucose with a mixed culture, it is found that the pH range 4.5–5.5 can provide good hydrogen yield. On the other hand, this research low hydrogen yields when using initial pH 5, possibly due to enzyme deactivation in the metabolic pathway favoring fermentative hydrogen production [51]. Initial pH 5 inhibited hydrogen production, while high initial pH 9 declined the lag phase but still yielded low hydrogen production [40,46]. During fermentation, the initial pH 7 at 35 °C was rapidly decreased to pH 5.4 in one day and then slightly increased to pH 5.6–6.2 by Day 6 of microalgae fermentation. The initial pH 7 at 35 °C of sucrose rapidly decreased in the first two days to nearly pH 5 and then dropped to pH 4.5 in Day 6. Inconstant, low pH 4.1–4.3 on Day 6 from the initial pH 5 was found for anaerobic fermentation of microalgae (Figure 4). The results indicate that the control of pH is important for hydrogen production [51]. In addition, at 35 °C, the decrease in pH from initial pH 5 and 7 was more rapid than fermentation at 55 °C. The reduction of pH was caused by acid accumulation in the fermentation system from the decomposition process of acid-producing

bacteria [12]. Especially, clostridia, one kind of acidogenic bacteria, can produce acid along with the production of hydrogen [13]. The reduction of pH demonstrates that hydrogen production occurred in the fermentation process. However, the decrease in low pH would greatly inhibit the metabolism of bacterial growth and cause a decrease in hydrogen production [52].

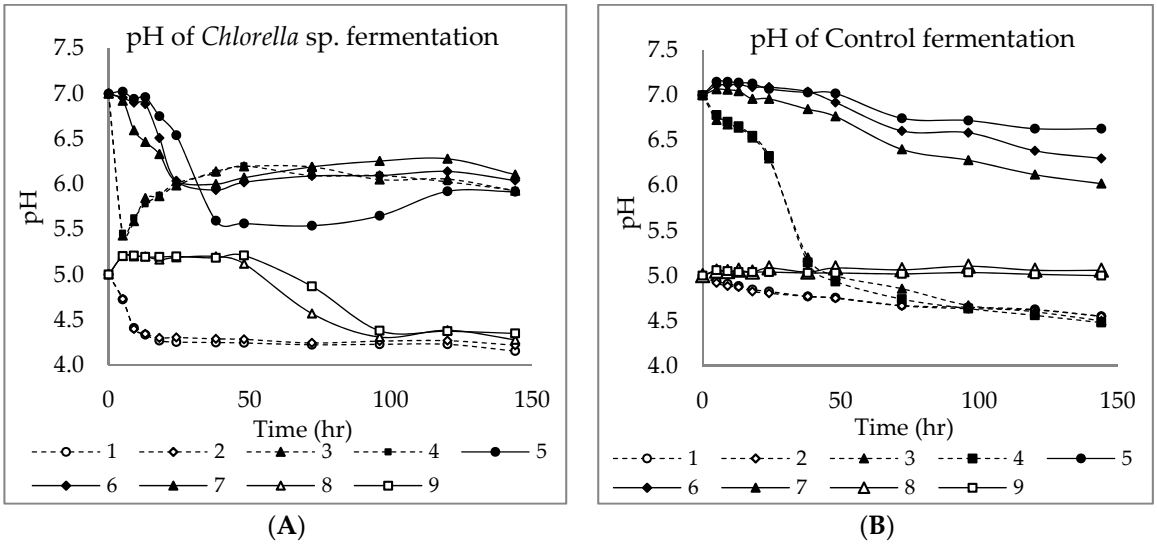


Figure 4. pH during fermentation in different batches: (**A**) *Chlorella* sp.; and (**B**) control.

3.3. Butyric Acid Production

Butyric acid can be used as a precursor for butanol production [9,12]. The results indicate that organic acid production from *Chlorella* sp. fermentation varied with the different initial concentrations, pH and temperatures by acidogenic mixed cultures. Figure 5 shows the production of butyric acid, propionic, and acetic acids at 35 °C and 55 °C. The fermentation process at the initial pH 5 showed that VFAs production had relatively low amounts, corresponding to very low hydrogen yield for both operating temperatures. On the other hand, fermentation at initial pH 7 and any concentration or temperature can produce VFAs with higher yield.

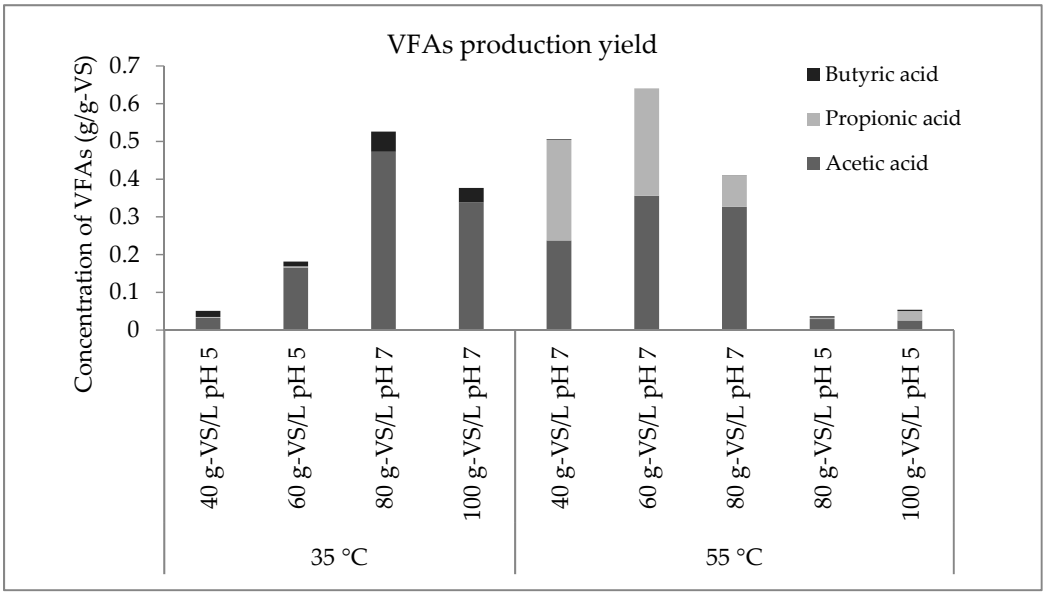


Figure 5. Acetic acid, propionic acid and butyric acid production from batch fermentation.

Acetic acid and butyric acid are the main products of the fermentation of microalgae at 35 °C, similar to the main products in the study by Giang et al. [53] at the same temperature, while, at operating temperature 55 °C, the main VFAs generated were acetic acid and propionic acid. High butyric acid production in batch fermentation was obtained at initial concentration of 80 and 100 g-VS/L at pH 7 with concentration of butyric acid production of 4.27 g/L (0.05 g/g-VS) and 3.81 g/L (0.04 g/g-VS), respectively (Table 3) .

Table 3. The hydrogen and VFAs product of *Chlorella* sp. fermentation by the Taguchi method.

Run	Conc. (g-VS/L)	pH	T (°C)	Acetic â (g/g-VS)	Propionic â (g/g-VS)	Butyric â (g/g-VS)	Total â (g/g-VS)	H_2 Yield (mL/g-VS)	% COD Removal
1	40	5	35	0.03	0.00	0.016	0.05	0.0	0.0
2	60	5	35	0.17	0.00	0.013	0.18	0.0	0.0
3	80	7	35	0.47	0.00	0.053	0.56	22.2	1.0
4	100	7	35	0.34	0.00	0.038	0.38	22.0	1.0
5	40	7	55	0.24	0.27	0.002	0.80	17.9	0.7
6	60	7	55	0.36	0.28	0.000	0.81	15.7	0.7
7	80	7	55	0.33	0.08	0.001	0.46	9.8	0.4
8	80	5	55	0.03	0.00	0.004	0.03	0.1	0.0
9	100	5	55	0.03	0.02	0.003	0.07	0.0	0.0

Butyric and acetic acids are obligatory produced along with hydrogen production. Carbohydrate monomer in the form hexose was directed to volatile fatty acids along with carbon dioxide and/or hydrogen, as shown in Reactions (1)–(3) [54]:

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 4H_2 + 2CO_2 + 2C_2H_4O_2 \quad (1)$$

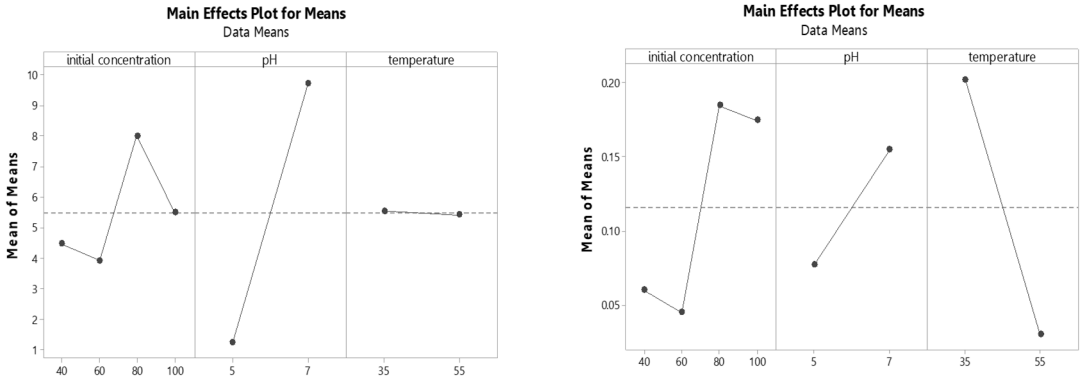
$$C_6H_{12}O_6 \longrightarrow 2H_2 + 2CO_2 + 2C_4H_8O_2 \quad (2)$$

$$C_6H_{12}O_6 + 2H_2 \longrightarrow 2C_3H_6O_2 + 2H_2O \quad (3)$$

Reaction (1): One mole of glucose can be converted to two moles of acetic acid concurrently with four moles of hydrogen production. Reaction (2): One mole of glucose can produce butyric acid along with only two moles of hydrogen. Reactions (1) and (2) can simultaneous produce butyric and acetic acids along with hydrogen. Reaction (3): Propionic acid production cannot occur simultaneously but two moles of hydrogen with one mole of glucose can further produce two moles of propionic acid. The temperature 55 °C produced high propionic acid yield in contrast with low hydrogen yield. The reaction for propionic acid does not produce hydrogen. The temperature of 35 °C produced lower total VFAs than 55 °C at initial pH 7, while yielding acetic acid and butyric acid as the main products. According to Giang et al. [53], when the total acid production is increased, the hydrogen production yield will decrease, as the hydrogen synthesis byproduct found in fermented solution contains mostly acetic acid and butyric acid [36]. Thus, the temperature of 55 °C yielded not only the highest acetic acid production but also propionic acid as a product, resulting in low hydrogen gas production because propionic acid is produced through a hydrogen consuming reaction [55].

In addition, the production of hydrogen and butyric acid under different conditions was analyzed by ANOVA in Minitab. The most significant ($P_{value} < 0.05$) factors for hydrogen and butyric acid production are pH ($P_{value} = 0.001$) followed by temperature ($P_{value} = 0.024$). Therefore, the main factors affecting butyric acid and hydrogen production in *Chlorella* sp. fermentation using mixed cultures were operating temperature and initial pH (Figure 6). In accordance with the review in [36], pH and temperature are the most crucial parameters for acidogenesis fermentation. In addition, the biomass used in the fermentation process is decomposed by microorganisms, transforming into volatile fatty acids, mixed carboxylate and cell mass, which remain in the system [17], but only COD contributed to hydrogen production is removed from the fermentation system [11,16]. Therefore, the hydrogen

yield obtained (Table 3) shows that there was a slight decrease in COD in the system. The hydrogen production yield from microalgae of Batch Sets 3 and 4 could be eliminated, being only about 1%, which represents that, after degradation, the decomposed matter remained in the system, especially in a volatile fatty acid form that can be used as a potential substrate to produce butanol in pure-culture ABE fermentation. Li et al. [9] used rice straw in the fermentation process to produce acids, which were subsequently used to produce butanol in ABE fermentation of *Clostridium beijerinckii* NCIMB 8052.



(**A**) (**B**)

Figure 6. Response table from three factors of initial concentration of substrate, pH and temperature for means of: (**A**) hydrogen production; and (**B**) butyric acid production.

4. Conclusions

The main factors affecting butyric acid production and hydrogen production were temperature and pH. The optimal conditions for production of hydrogen and butyric acid from *Chlorella* sp. using mixed acidogenic bacteria were: 35 °C, pH 7 and concentration of 80–100 g-VS/L. The average hydrogen production yield of 22 mL-H_2/g-VS and butyric acid production yield of 0.05 g/g-VS were obtained, suggesting that microalgae (*Chlorella* sp.) has potential to be converted directly to butyric acid by using acidogenesis under above optimum conditions. Moreover, mixed cultures fermentation could be helpful to produce potentially butyric acid from microalgae without any pretreatment and without adding nutrients. Butyric acid could be valuable substrate for further ABE fermentation by pure culture to produce butanol, an advanced biofuel.

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