



Research article

Buffering action of acetate on hydrogen production by *Ethanoligenens harbinense* B49



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ABSTRACT

The buffering effect of acetate on hydrogen production during glucose fermentation by *Ethanoligenens harbinense* B49 was investigated compared to phosphate, a widely used fermentative hydrogen production buffer. Specific concentrations of sodium acetate or phosphate were added to batch cultures, and the effects on hydrogen production were comparatively analyzed using a modified Gompertz model. Adding 50 mM acetate or phosphate suppressed the hydrogen production peak and slightly extended the lag phase. However, the overall hydrogen yields were 113.5 and 108.5 mmol/L, respectively, and the final pH was effectively controlled. Acetate buffered against hydrogen production more effectively than did phosphate, promoting cell growth and preventing decreased pH. At buffer concentrations 100–250 mM, the maximum hydrogen production was barely suppressed, and the lag phase extended past 7 h. Therefore, although acetate inhibits hydrogen production, using acetate as a buffer (like phosphate) effectively prevented pH drops and increased substrate consumption, enhancing hydrogen production.

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1. Introduction

Research into alternative energy sources has attracted renewed interest following an increased global awareness of accumulated CO₂ in the atmosphere and its role as a potential cause of climate change [1]. Hydrogen is an ideal clean and sustainable energy source that can be used in fuel cells, transportation and other industries. Compared with conventional hydrogen production processes, including the electrolysis of water, the reforming of natural gas and oil and the gasification of coal, biological hydrogen production offers a promising technique that makes use of renewable biomass and organic wastewater.

Biohydrogen production can be divided into two main categories: hydrogen production by photosynthetic organisms using light and hydrogen production via fermentative metabolism by anaerobic bacteria [2,3,4]. Relative to the photosynthetic production of hydrogen, fermentative processes offer the advantages of higher hydrogen production rates without illumination and the ability to convert organic wastes into more valuable energy sources.

Many factors [5], such as the carbon source [6,7,8], nitrogen source [9,10], hydrogen pressure [11,12,13], pH [14], temperature [15] and

end-products [16], can influence fermentative hydrogen production. Among these factors, pH is one of the factors controlling anaerobic biological processes [17]. In an anaerobic reactor the pH value and its stability are important. This pH value and stability are relevant to different acid–base systems such as propionic, butyrate and mixed fatty acid systems. The formation of hydrogen is accompanied with volatile fatty acids (VFAs) or solvents during the anaerobic digestion process. The accumulation of these acids causes a sharp decrease in the culture pH and subsequently inhibits bacterial hydrogen production, a failure to control pH changes due to volatile fatty acid (VFA) imbalances can interrupt hydrogen production [17,18]. Buffers and automatically controlled pH systems are two commonly used methods for this purpose in anaerobic hydrogen production systems. Because of their convenience and availability, carbonate and phosphate are two important components in acid–base buffer systems and are widely used in anaerobic hydrogen production systems [19]. Phosphate in particular is regarded as an appropriate buffer, and its effects on hydrogen production by *Ethanoligenens harbinense* B49 have already been studied [20].

E. harbinense B49 was isolated from a continuous flow, high-rate acidogenic reactor using ethanol-type fermentation, and it is a Gram-positive, mesophilic, strictly anaerobic bacterium that is phylogenetically related to the clostridia class. This bacterium is one of the most promising producer organisms due to its capability to

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efficiently and rapidly generate hydrogen [11], and its characteristics make it an interesting target for physiological and genetic studies aiming to improve its metabolic properties and increase its productivity with respect to hydrogen. This microorganism produces ethanol as a major fermentation product, in addition to CO₂, acetate and H₂ [21,22,23]. The addition of ethanol had little inhibitory effect on fermentative hydrogen production, and the addition of acetate had a strong inhibitory effect on glucose consumption, bacterial growth and hydrogen production of *E. harbinense* B49 [24]. The hydrogen production is affected by the accumulation of self-produced byproducts, and acetate is therefore regarded as having an inhibitory rather than a buffering action during fermentative hydrogen production by *E. harbinense* B49. The inhibitory effect of acetate has been studied to understand the hydrogen-producing characteristics of these cultures, but its buffering effect has not been explored. In this paper, the effects of acetate on hydrogen production by *E. harbinense* B49 were investigated to examine the buffering action of acetate on this process.

2. Materials and methods

2.1. Microorganism and media

The hydrogen-producing strain *E. harbinense* B49 (AF481148 in EMBL) was isolated from a continuous flow, high-rate acidogenic reactor using ethanol-type fermentation and then identified as a novel *Ethanoligenens* strain [24]. The strain was stored in our lab at –80°C and cultured at 36°C at an initial pH of 6.5 under strict anaerobic conditions. Cells from stock cultures were transferred into 50-mL volumes of sterilised growth medium and incubated at 35°C. When the cells entered a logarithmic growth phase, 5 mL of the pre-cultured broth was inoculated into a 100-mL serum bottle containing 50 mL of basal medium, and the culture was grown anaerobically at 35°C with shaking at 130 rpm. The hydrogen production medium consisted of (in g/L): glucose 10.0, yeast extract 3.0, NH₄Cl 0.5, MgCl₂ 0.18, K₂HPO₄ 1.5, NaH₂PO₄ 4.2 and L-cysteine 0.5. The basal medium also contained 1% trace element solution, 1% vitamin solution and 0.2% resazurin. The cells were harvested at the end of the exponential phase and used as inocula for the batch experiments.

2.2. Batch tests

The buffering activities and inhibitory effects of phosphate, acetate and ethanol on the hydrogen-producing performance of strain B49 were investigated using serum bottles as batch reactors. All of the batch-fermentation studies were performed in 250-mL serum bottles with a 120-mL working volume. The hydrogen production medium also contained sodium acetate (NaAc·3H₂O, at 0, 50, 100, 150, 200 or 250 mM) or phosphate (Na₂HPO₄ × 2H₂O-KH₂PO₄, at 0, 50, 100, 150, 200 or 250 mM). Three bottles were tested in parallel for each condition. All media were sterilised by autoclaving at 121°C and 15 psig for 30 min. Each bottle was then inoculated with 5.0 mL of strain B49 cell suspension and incubated under non-controlled pH

conditions in an air-bath shaker at 36 ± 1°C and 135 rpm. The biogas was sampled for biogas content analysis using a syringe, and a liquid sample was simultaneously taken from the bottles. All tests were run in triplicate.

2.3. Analytical methods

2.3.1. Cell growth analysis

The cell dry weight was determined by drying the cells for 24 h at 80°C to a constant weight in a convection-type hot air oven (HPG-9145, China).

2.3.2. Liquid samples

Cells in the liquid cultures were pelleted by centrifugation at 8000 rpm for 5 min at room temperature. The culture supernatant was filtered through a 2.5-cm diameter, 0.45-µm polytetrafluoroethylene filter, transferred to sterile 1-mL Eppendorf tubes and frozen until analysis. Volatile fatty acids and ethanol were detected using a gas chromatography (GC) system (HP 6890, Agilent Technologies, USA) and a flame-ionisation detector (FID). The temperatures of the glass columns and injections were 145°C and 175°C, respectively. The carrier gas was N₂, and the packing material was FON (which contains polyethylene glycol and 2-nitroterephthalic acid), obtained from Shimadzu, Inc. The glucose concentration in the culture was determined according to the protocol in a kit (GOD-PAP, Shanghai Rongsheng Biological Technology Corporation, China), and the pH was measured using a PHS-25 acidity voltmeter according to standard methods.

2.3.3. Biogas composition

Biogas production was measured using the water displacement method. The biogas composition from the bioreactor was measured using GC (HP 4890, Agilent) on an instrument equipped with a thermal conductivity detector (TCD). A stainless steel column packed with molecular sieve 5 A was used to detect H₂. Nitrogen was used as the carrier gas at a rate of 25 mL/min.

3. Modeling the kinetic parameters

The cumulative hydrogen production data were fitted using a modified Gompertz equation [25,26] as a suitable model for describing the progress of cumulative hydrogen production in the batch experiment.

$$H = P \cdot \exp\left\{-\exp\left[\frac{R_m}{P}(\lambda - t) + 1\right]\right\} \quad [\text{Equation 1}]$$

in which H is the cumulative hydrogen production (in mL/L); P is the hydrogen production potential (in mL/L); R_m is the maximum hydrogen production rate (as mL/L/h); λ is the time of the lag phase (h); e is 2.7182; and t is the incubation time (h).

Table 1
Glucose degradation, cell growth and terminal pH at various acetate or phosphate concentrations.

Buffer con. (mM)	Degradation rate (%)		Biomass (mg/L)		Terminal pH	
	Phosphate	Acetate	Phosphate	Acetate	Phosphate	Acetate
0	97 ± 1.2	97 ± 1.2	619.32 ± 61.9	623 ± 61.5	3.75 ± 0.03	3.75 ± 0.03
50	100 ± 1.4	100 ± 1.1	646.07 ± 58.3	916 ± 75.4	4.15 ± 0.03	4.73 ± 0.03
100	95 ± 0.6	100 ± 0.6	616.87 ± 46.5	1044 ± 71.4	4.60 ± 0.03	5.07 ± 0.03
150	87 ± 1.1	100 ± 0.9	505.68 ± 56.4	1138 ± 61.2	5.35 ± 0.03	5.22 ± 0.03
200	63 ± 1.6	100 ± 1.4	455.19 ± 50.3	1166 ± 80.3	6.05 ± 0.03	5.33 ± 0.03
250	50 ± 0.5	98 ± 0.8	420.24 ± 44.9	1213 ± 90.1	6.20 ± 0.03	5.41 ± 0.03

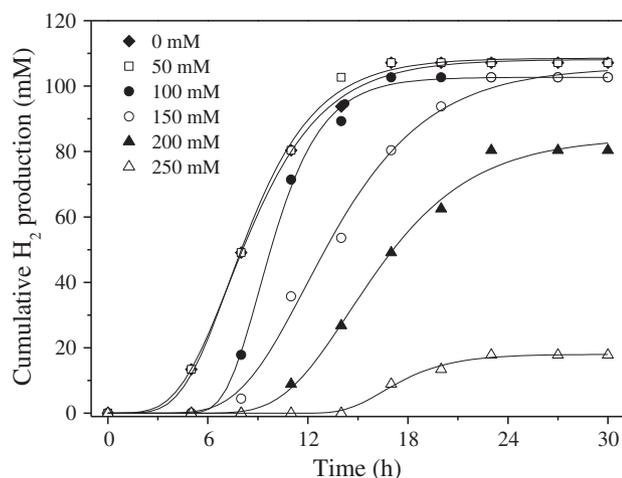


Fig. 1. Time course of hydrogen production profiles during fermentation of glucose under different phosphate concentration conditions. The lines represent data calculated using Gompertz equation.

4. Results and discussion

4.1. Glucose degradation and cell growth

The glucose degradation efficiencies, cell growth and terminal pH values at various phosphate and acetate concentrations are illustrated in Table 1. In the tests of acetate, the glucose was almost completely degraded at the end of fermentation, achieving 97–100% total glucose degradation. In contrast to acetate, the addition of phosphate had an obvious inhibitory effect on glucose degradation. From 0 to 50 mM phosphate, the glucose degradation reached approximately 97–100%. When more phosphate was added beyond 100 mM, the glucose degradation rates declined, achieving only 50% glucose degradation at 250 mM. This result indicates that the addition of excess phosphate had a significant negative influence on glucose degradation.

In the phosphate tests, cell growth was improved with 50 mM phosphate. However, as the phosphate concentration increased, cell growth was gradually inhibited. In contrast, the total cell weight increased as more acetate was added. This result was inconsistent with that of another research report, a discrepancy that may be due to differences in the composition of the hydrogen production medium [24]. The initial pH in all tests was approximately 6.5. As shown in Table 1, at the end of hydrogen-producing fermentation, the terminal pH values of media supplemented with 50 mM or 100 mM phosphate were much higher than those of acetate-supplemented media. However, when the concentrations of phosphate and acetate exceeded 150 mM, the terminal pH values of the acetate-supplemented media were much higher than those supplemented with phosphate, due to the buffer system of sodium acetate and acetate. These results indicated that acetate was able to promote the cell growth of *E. harbinense* B49 and raise the terminal pH as a result of its enhanced buffering of the fermentative system. In contrast, although phosphate

was able to raise the terminal pH by buffering $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O} - \text{KH}_2\text{PO}_4$, it also restrained cell growth.

4.2. Time course of hydrogen production profiles

4.2.1. Under different phosphate concentration conditions

Hydrogen production by *E. harbinense* B49 was significantly affected by the phosphate concentration of the medium. As shown in Fig. 1 and Table 2, a slight increase in the cumulative hydrogen yield could be achieved by increasing the phosphate buffer concentration from 0 mM to 50 mM. A maximum P_{max} of 108.54 mmol/L and R_{max} of 18.39 mmol/L/h were observed at phosphate buffer concentrations of 50 mM and 100 mM, respectively. Subsequently, P_{max} and R_{max} decreased gradually as the phosphate buffer concentration increased, most likely due to the negative effect of increased cytoplasmic osmotic pressure [27].

The lag phase times of hydrogen production became longer as the phosphate concentration increased. The final pH also increased with increasing phosphate buffer concentrations, whereas lower phosphate buffer concentrations were associated with lower pH values. Similar to glucose consumption and cell growth, hydrogen production also peaked at 50 mM phosphate, as shown in Table 1 and Table 2.

Different results were obtained in previous studies of *Citrobacter* sp. Y19 [28] and *Rhodopseudomonas palustris* P4 [29]. No inhibitory effect of phosphate on cell growth was observed at concentrations between 0 and 300 mM. The maximum hydrogen yield was obtained at concentrations of 50 and 140 mM phosphate by *R. palustris* P4 and *Citrobacter* sp. Y19, respectively. The present results indicate that the optimal phosphate concentration is 50 mM for *E. harbinense* B49. At this concentration, the maximal yield of hydrogen was produced; the most glucose was exhausted; and the lag phase was relatively shorter. Similar results were reported for *Clostridium beijerinckii* Fanp3 [30].

4.2.2. Under different acetate concentration conditions

The effects of acetate concentration on hydrogen production are shown in Fig. 2 and Table 2. The glucose in the media was completely exhausted after 48 h of incubation irrespective of the acetate concentration.

However, the addition of acetate had a considerable impact on the cumulative hydrogen production. Compared with hydrogen production medium that did not include acetate, an increase in the cumulative hydrogen yield could be achieved by increasing the acetate buffer concentration to 50 mM, and the addition of additional acetate extended the lag phase of hydrogen production. The maximum P_{max} of 113.53 mmol/L and R_{max} of 12.56 mmol/L/h occurred at an acetate buffer concentration of 50 mM and in media with no acetate added, respectively. When the concentration of acetate was greater than 50 mM, slight inhibition of hydrogen production occurred, and the P_{max} and R_{max} values decreased gradually with increasing acetate concentration. However, cell growth was inversely related to the hydrogen production rate and increased with increasing acetate, as shown in Table 1.

Table 2

Fermentation characteristics for hydrogen production at various phosphate or acetate concentrations.

Buffer con. (mM)	P_{max} (mmol/L)		R_{max} (mmol/L/h)		λ (h)		R^2	
	Phosphate	Acetate	Phosphate	Acetate	Phosphate	Acetate	Phosphate	Acetate
0	108.14	108.14	12.84	12.56	4.2	4.2	0.9986	0.9986
50	108.54	113.53	13.96	10.53	4.4	6.8	0.9979	0.9905
100	102.69	106.52	18.39	6.19	7.0	8.8	0.9984	0.9932
150	105.86	103.84	9.69	6.30	7.9	11.4	0.9955	0.9988
200	84.34	104.02	7.74	6.16	10.5	11.6	0.9959	0.9971
250	18.01	100.22	2.96	7.45	14.2	13.4	0.9944	0.9941

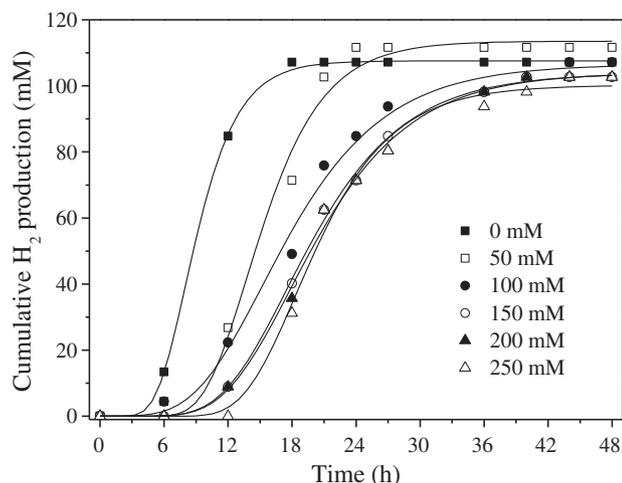


Fig. 2. Time course of hydrogen production profiles during fermentation of glucose under different acetate concentration conditions. The lines represent data calculated using Gompertz equation.

4.3. The amount of volatile organic compound and hydrogen

The amount of acetate, ethanol and hydrogen at various phosphate and acetate concentrations are shown in Fig. 3. The amount of ethanol were increased slightly with increasing phosphate or acetate concentration, the concentration of acetate and the volume of hydrogen were varied only slightly while the concentration of acetate was increased, but decreased dramatically while the concentration of phosphate was increased. In contrast to acetate, the addition of phosphate had an obvious inhibitory effect on acetate and hydrogen production. At 250 mM acetate, the volume of hydrogen exceeded 100 mM which achieved the maximum volume of hydrogen at 50 mM acetate. However, at 250 mM phosphate, the volume of hydrogen was less than 20 mM which is one fifth of the maximum volume of hydrogen at phosphate. This result indicates that the addition of excess phosphate had a significant negative influence on hydrogen production.

Hydrogen production from glucose by hydrogen-producing microorganisms also yields volatile organic acids, such as acetic acid and butyric acid, which lower the pH of the media and slow hydrogen

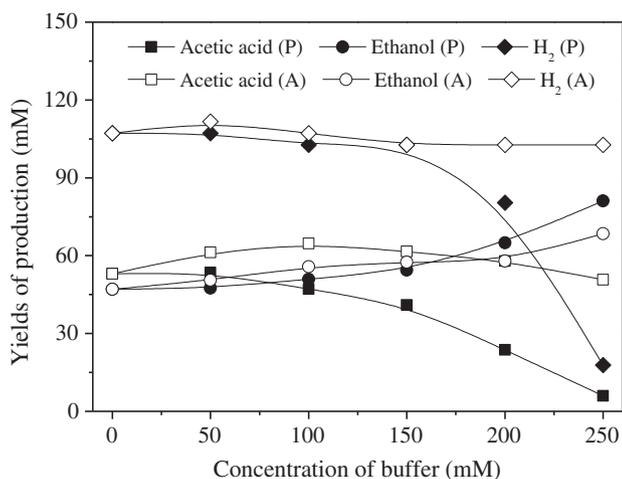


Fig. 3. The change of acetate, ethanol and hydrogen yield under different phosphate or acetate concentration conditions (P and A represented phosphate and acetate buffer, respectively). The yield of acetate subtracted the concentration of acetate-supplemented in the media under different acetate concentration conditions.

production [31]. To minimise the effects of these organic acids on the pH, phosphate buffers composed of Na_2HPO_4 and NaH_2PO_4 or KH_2PO_4 were used to control the pH. Acetic acid is mainly a product of fermentation; therefore, acetate has been regarded as an inhibitor of hydrogen production and has not been used to control pH. However, the present results indicate that acetate was able to control the pH during fermentative hydrogen production from glucose by *E. harbinense* B49. We also evaluated the ability of phosphate and acetate to control pH during fermentation, and we found that although both phosphate and acetate were able to control the pH through their buffering activity, acetate was a stronger buffer than phosphate until the concentrations exceeded 150 mM. The final pH increased with increasing concentrations of acetate and phosphate, but their patterns of buffer activity may be different. Sodium acetate and acetate, which were produced during fermentative hydrogen production, formed a buffer that grew increasingly strong. Acetate was “internal buffer system”, while phosphate was “external buffer system”.

5. Conclusions

The addition of acetate had both inhibitory and buffering effects on hydrogen production from glucose by *E. harbinense* B49. Acetate was able to control the pH changes caused by fermentative hydrogen production and increased the yield of hydrogen. At an acetate concentration of 50 mM, maximal hydrogen production of 113.5 mmol/L was achieved. The inhibitory effect of acetate on hydrogen production was mainly due to an extended lag phase, and acetate slightly decreased the cumulative hydrogen volume when added at concentrations between 100 and 250 mM. Therefore, using acetate as a buffering supplement can control the pH and alleviate the acidification of the growth medium.

Conflict of interest

We have no conflict of interest to declare.

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