J. Microbiol. Biotechnol. (2008), **18**(6), 1136–1140

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Extracellular 5-Aminolevulinic Acid Production by *Escherichia coli* Containing the *Rhodopseudomonas palustris* KUGB306 *hemA* Gene

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Received: September 4, 2007 / Accepted: December 5, 2007

The *Rhodopseudomonas palustris* KUGB306 *hemA* gene codes for 5-aminolevulinic acid (ALA) synthase. This enzyme catalyzes the condensation of glycine and succinyl-CoA to yield ALA in the presence of the cofactor pyridoxal 5'-phosphate. The *R. palustris* KUGB306 *hemA* gene in the pGEX-KG vector system was transformed into *Escherichia coli* BL21. The effects of physiological factors on the extracellular production of ALA by the recombinant *E. coli* were studied. Terrific Broth (TB) medium resulted in significantly higher cell growth and ALA production than did Luria-Bertani (LB) medium. ALA production was significantly enhanced by the addition of succinate together with glycine in the medium. Maximal ALA production (2.5 g/l) was observed upon the addition of D-glucose as an ALA dehydratase inhibitor in the late-log culture phase. Based on the results obtained from the shake-flask cultures, fermentation was carried out using the recombinant *E. coli* in TB medium, with the initial addition of 90 mM glycine and 120 mM succinate, and the addition of 45 mM D-glucose in the late-log phase. The extracellular production of ALA was also influenced by the pH of the culture broth. We maintained a pH of 6.5 in the fermenter throughout the culture process, achieving the maximal levels of extracellular ALA production (5.15 g/l, 39.3 mM).

Keywords: Extracellular production, 5-aminolevulinic acid, *Escherichia coli*, *Rhodopseudomonas palustris*, *hemA*

5-Aminolevulinic acid (ALA) is a precursor of the tetrapyrroles, which include heme, porphyrin, chlorophyll, and vitamin B_{12}, and are synthesized *via* porphobilinogen. ALA is a key metabolic intermediate in the regulation of tetrapyrrole biosynthesis [7, 27]. ALA can be biosynthesized through two major pathways [6]. One is the C_4 pathway, which is present in mammals, birds, yeast, some protozoa, and purple nonsulfur photosynthetic bacteria. In the C_4 pathway, the pyridoxal 5'-phosphate-dependent enzyme ALA synthase [ALAS; succinyl-CoA: glycine succinyl transferase (decarboxylating), E.C. 2.3.1.37] catalyzes the condensation of succinyl-CoA and glycine to yield ALA. The second route is the C_5 pathway, which occurs in higher plants, algae, and in many bacteria, including *Escherichia coli* [12] and the archaea. In the C_5 pathway, ALA is formed from glutamate *via* three enzymatic steps. Recently, ALA has received a great deal of attention, largely due to its potential use as an herbicide, insecticide, antimicrobial drug, and photosensitizer for photodynamic therapy [11, 14, 17, 18, 20]. However, until recently, mass production of ALA was a difficult proposition, owing to the numerous steps required for its chemical synthesis [25]. Therefore, many studies have sought to develop an improved method for the biological production of ALA. Using a *Rhodobacter sphaeroides* mutant selected after nitrosoguanidine treatment, ALA was obtained at 20 mM in 42 h *via* aerobic dark fermentation [16]. However, strain improvement by mutation and screening is inefficient and time-consuming. Recently, most researchers have preferred metabolic engineering to mutation for improving ALA production. Various recombinant strains of *E. coli*, which lacks native ALAS, harboring the *hemA* gene mainly from *R. sphaeroides* have been engineered, and their abilities to produce ALA have been investigated [1, 4, 23, 26, 28, 29] (Table 1). Using factorial design optimization to improve ALA synthase activity, the yield of ALA reached 5.2 g/l using recombinant *E. coli* containing the *R. sphaeroides hemA* gene [29].

Previously, we have cloned and sequenced the *hemA* gene encoding for ALAS from *Rhodopseudomonas palustris* KUGB306, and characterized the properties of the recombinant

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MATERIALS AND METHODS

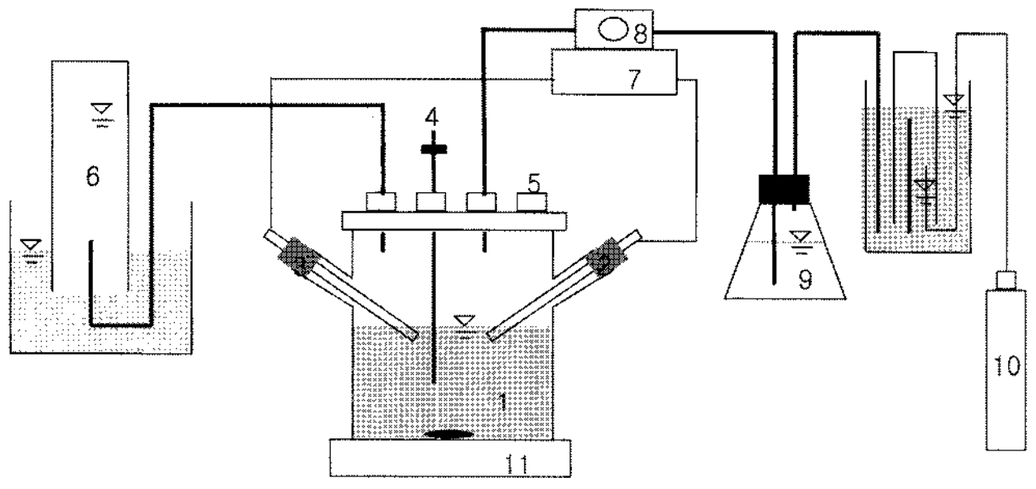
Seed Microorganisms

Sludge was originated from the return sludge line of a sewage water treatment plant of Uijeongbu (Korea). The sludge was settled for 30 min to remove suspended solids. A synthetic medium containing 1 g of sucrose per 100 ml was placed into a serum bottle. The initial pH of the medium was 7.0. The medium in the serum bottle was purged with pure nitrogen gas for 5 min to obtain anaerobic conditions. The medium was inoculated with 6 ml of the supernatant of the settled sludge and then continuously shaken at 120 rpm on a mechanical shaker at 30°C. The gas produced in the headspace of the serum bottle was collected and analyzed to measure the amount of hydrogen produced. Serial transfers of the cultures were conducted with the fresh medium in the serum bottles.

The morphologies of hydrogen-producing bacteria in the serum bottle were analyzed by scanning electron microscopy (SEM). The microbial community is composed of various bacilli of different lengths (Fig. 1A). In order to analyze the complexity of the hydrogen-producing microbial community, DNA of the microorganisms in the serum bottle was extracted. The 16S rDNA fragments were amplified by a polymerase chain reaction (PCR) using 341f and 518r as primers [25] and then separated by denaturing gradient gel electrophoresis (DGGE). Fig. 1B illustrates the DGGE profiles of the 16S rDNA gene fragment amplified from the microorganisms. Each band on the DGGE profile corresponds to a gene fragment of unique 16S rDNA sequences, representing specific species in the microbial community. The bands (designated as B1, B2, B3, and B4) of the profile were identified as *Pantoea agglomerans*, *Clostridium* sp., *Enterobacter* sp., and *Enterobacter cloacae* by BLAST program analysis (Fig. 1B). *Clostridium* sp., *Enterobacter* sp., and *Enterobacter cloacae* were reported as being hydrogen-producing bacteria in previous studies [3, 6, 19].

Operation of Batch Reactor

Hydrogen production experiments were conducted in a 1.5-l batch fermentor at 30±3°C (Fig. 2). The incubation solution contained (per liter) 15 g of sucrose, 2 g of NH_4HCO_3, 1 g of KH_2PO_4, 100 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of NaCl, 10 mg of $NaMoO_4 \cdot 2H_2O$, 10 mg of $CaCl_2 \cdot 2H_2O$, 15 mg of $MnSO_4 \cdot 7H_2O$, and 2.78 mg of $FeCl_2$.



1. Culture medium
2. pH meter sensor
3. Temperature sensor
4. Liquid sampling port
5. Gas sampling port
6. Biogas collector
7. pH controller
8. Pump
9. 2N-KOH solution
10. N_2
11. Magnetic stirrer

Fig. 2. Schematic diagram of the batch reactor for anaerobic fermentative hydrogen production.

The batch fermentor was stirred at a constant rate of 250 rpm by a magnetic stirrer and the headspace was initially filled with nitrogen gas. The pH of the solution was automatically controlled by adding a 2 N aqueous KOH solution *via* a peristaltic pump. The reactor was kept in the dark by wrapping with aluminum foil, thus preventing the growth of photosynthetic bacteria and algae. The initial pH of the solution in the reactor was changed stepwise from 3.0 to 10.0 with the increment of 1.0. After the establishment of the reactor, a seed solution of 15 ml from the serum bottle was added to the reactor. The initial concentration of the VSS after adding the seed solution to the reactor was approximately 6 mg/l. The produced gas was collected in the biogas collector filled with 2% aqueous H_2SO_4 (v/v) solution. The gas volume was measured and gas aliquots of 0.5 ml were periodically withdrawn from the headspace of the reactor at regular intervals for analysis.

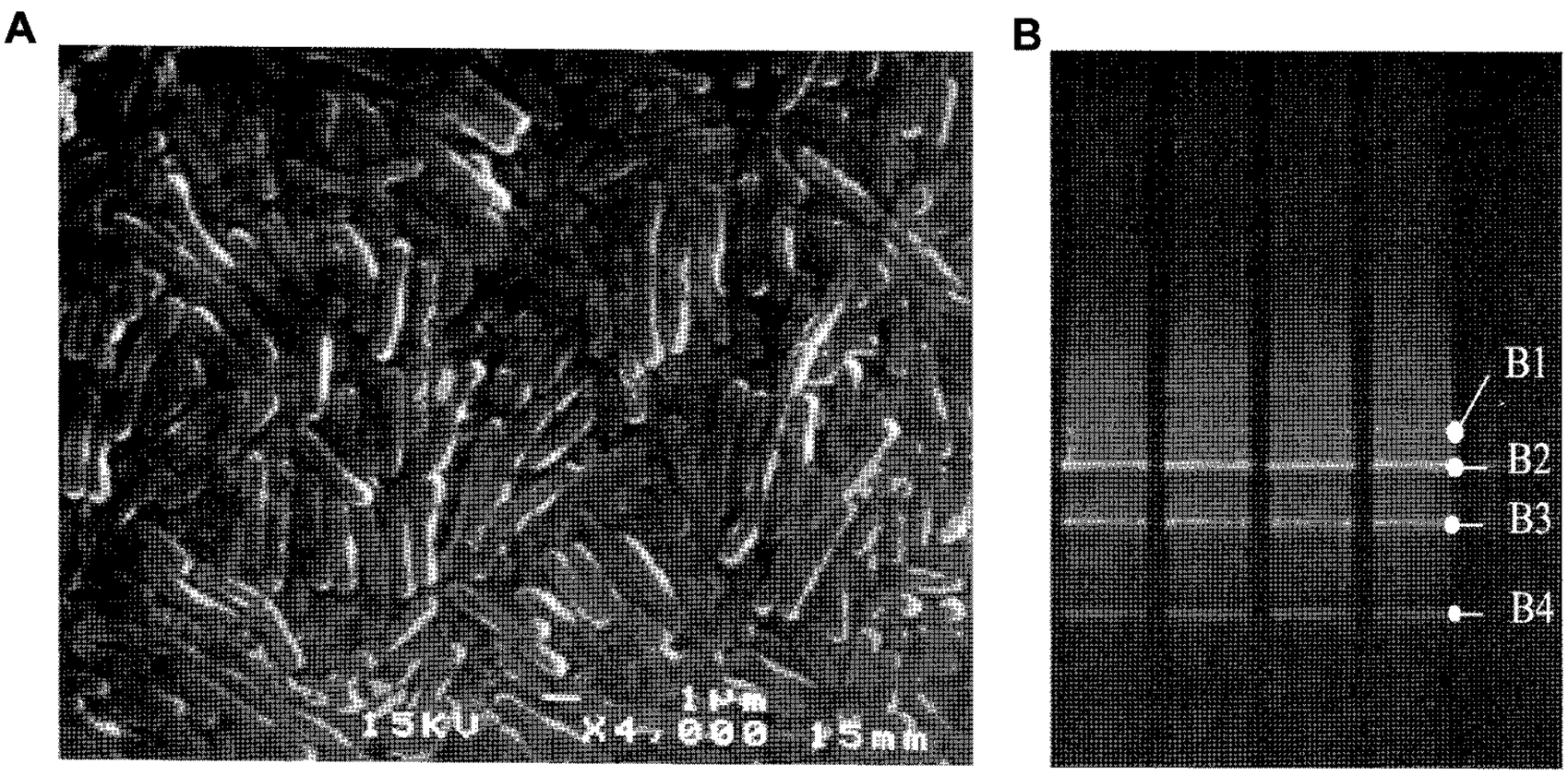


Fig. 1. Morphologies (**A**) and DGGE profiles (**B**) of the microorganisms in the serum bottle.
B1, B2, B3, and B4 on (**B**) were identified as *Pantoea agglomerans* (Similarity=94%), *Clostridium* sp. (Similarity=90%), *Enterobacter* sp. (Similarity=98%), and *Enterobacter cloacae* (Similarity=99%), respectively. The results of the sequence affiliation were determined by the BLAST program.

Analytical Methods

The composition of biogas in the headspace of the fermentor was determined with a gas chromatography system (GOW Mac Series 350) equipped with a thermal conductivity detector (TCD). Chromatographic separation of the reactor headspace samples was achieved by using a 6'×1/8" stainless steel SS 350A Molsieve 13X (80/100 mesh) with nitrogen as the carrier gas at a flow rate of 30 ml/min. The operation temperature of the injection port, oven, and detector were 80°C, 100°C, and 100°C, respectively.

The concentration of volatile suspended solids (VSS) and chemical oxygen demand (COD) were determined according to standard procedures [2]. Carbohydrate concentration was measured by the phenol-sulfuric acid method [8]. The fatty acids (acetic acid, butyric acid, formic acid, and propionic acid) were analyzed using a high-performance liquid chromatography (HPLC) system (KNAUER, German) equipped with a UV detector operated at 210 nm and Supelcogel C-610H (130 mm×7.8 mm ID).

Model Development

A modified Gompertz equation (Eq. 1) was used to fit the cumulative hydrogen production curves for each experiment to obtain a hydrogen production potential (P) and a hydrogen production rate (R) [15, 16].

$$M=P\exp\left\{-\exp\left[\frac{Re}{P}(\lambda-t)+1\right]\right\} \quad (1)$$

In Eq. (1), M is the cumulative hydrogen production (in ml), λ is the lag-phase time (h), P is the hydrogen production potential (ml), R is the maximum hydrogen production rate (ml/h), t is the incubation time (h), and e is the exp (1)=2.718. Parameters were estimated using the curve fit function in Sigma Plot 2002 (SPSS Science, U.K.). The specific gas production, R_h (ml/g VSS-h), was calculated by dividing R by the mass of the VSS in the reactor.

The effect of the hydrogen ion concentration on the specific hydrogen production rate is described by a modified Haldane equation (Eq. 2), assuming that the proton ion concentration is a limiting factor of hydrogen production.

$$R_h=\frac{R_m[H^+]}{\left[[H^+]+K_{OH}+\frac{[H^+]^2}{K_H}\right]} \quad (2)$$

In Eq. (2), R_m is the maximum specific hydrogen production rate, and K_{OH} and K_H are rate constants. This equation has been applied to nitrifying bacteria, lactate fermentation, and heterotrophic bacteria [1, 17, 30]. A nonlinear regression was used to fit the model described by Eq. (2) between pH 4.0 and pH 9.0 for the specific hydrogen production rate in the reactor. The best values of kinetic parameters, R_m, K_{OH}, and K_H, were determined using a curve fit function in Sigma Plot 2002 (SPSS Science, U.K.). The optimal pH value for hydrogen production was evaluated as $(pK_{OH}+pK_H)/2$, where pK_{OH} and pK_H are the negative logarithm of the constants K_{OH} and K_H, respectively.

Results and Discussion

Effect of pH on Sucrose Degradation

Fig. 3 shows the efficiency of sucrose degradation at several pH values. Sucrose degradation was not observed at pH 3.0 or 10. A sucrose degradation efficiency of 100% was observed between pH 5.0 and pH 9.0. The degradation rate decreased with increasing pH values between 6.0 and 9.0. A degradation efficiency of only 34% was observed at pH 4 after the 100 h incubation period. Minimal incubation time was required for the complete degradation of the sucrose at pH 6. Considering the results of the sucrose degradation, it shows that the value of pH is extremely important because relatively faster degradation was observed in the optimal pH.

Effect of pH on Hydrogen Production

The gases detected from the anaerobic fermentation process in this study were hydrogen and carbon dioxide; no methane

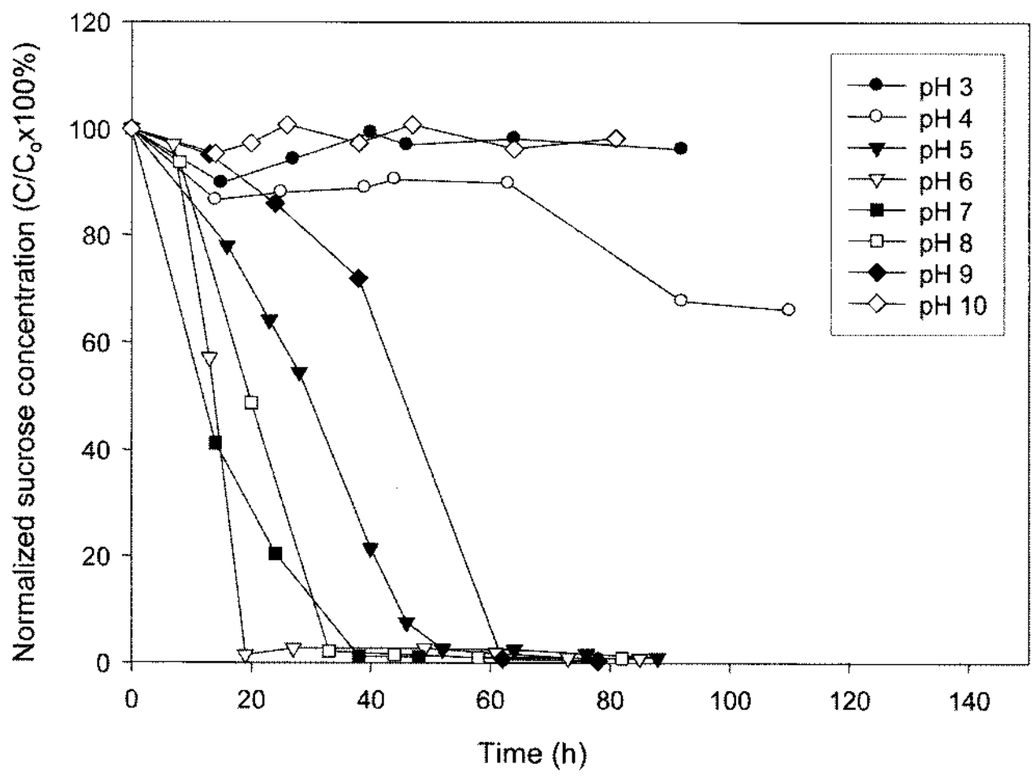


Fig. 3. Effect of pH on the sucrose degradation by a mixed microbial flora.

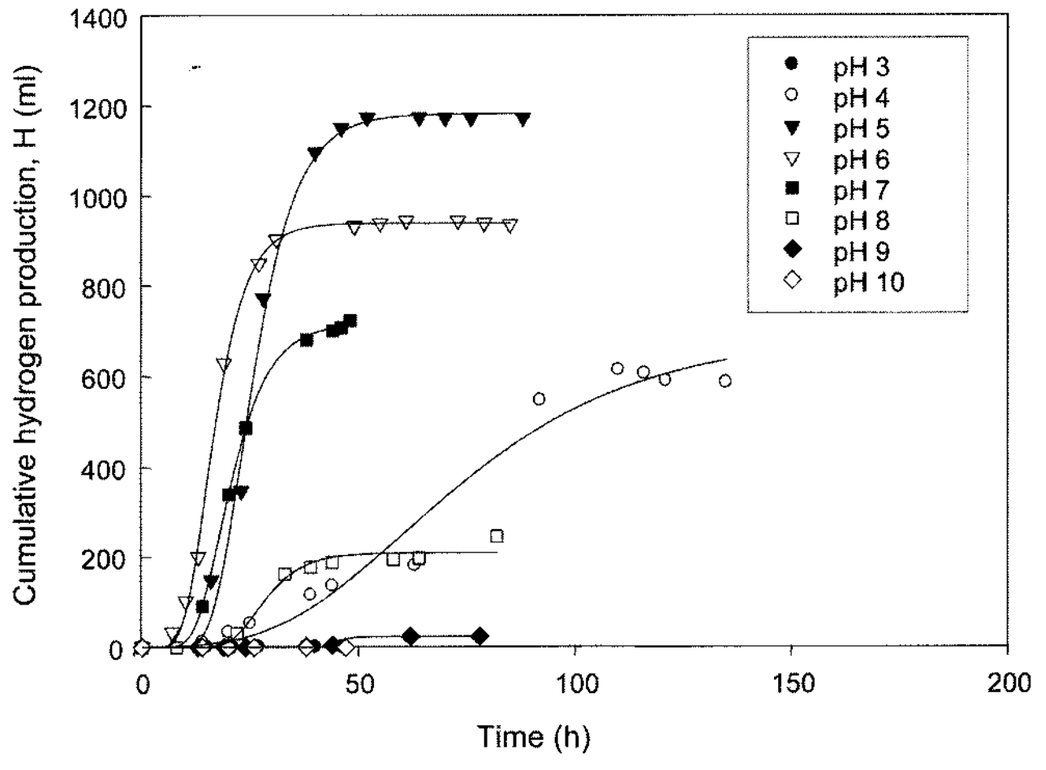


Fig. 4. Cumulative hydrogen production curve at various pH values.
The curves are the nonlinear estimation results according to Eq. (1).

Table 1. Calculated parameter values from nonlinear regression of Eq. (1) for hydrogen production.

pH	Hydrogen production potential		H_2 yield (ml/g COD)	Biomass (mg VSS/l)	Hydrogen conversion ratio (%)	Specific hydrogen production rate		r^2
	P_h (ml)	*p*-Value				R_h (ml/g VSS-h)	*p*-Value	
3	-	-	-	-	-	-	-	-
4	688.35	<0.0001	60.55	141.23	13.15	60.56	0.0002	0.98
5	1,182.09	<0.0001	117.56	569.08	22.56	112.46	<0.0001	0.99
6	939.25	<0.0001	88.48	626.72	17.93	107.59	<0.0001	0.99
7	714.21	<0.0001	67.96	674.84	13.18	64.73	<0.0001	0.99
8	209.64	<0.0001	19.68	607.14	3.97	21.44	<0.0001	0.98
9	24.13	<0.0001	2.12	453.94	0.46	10.71	<0.0001	0.99
10	-	-	-	-	-	-	-	-

was detected at the entire pH range tested. The pH had significant effects on both hydrogen production potential and hydrogen production rate (Fig. 4). Maximum hydrogen production was obtained at pH 5.0.

To better understand the pH effect on hydrogen production, the hydrogen production potential (P) and maximum hydrogen production rate (R_h) obtained from the modified Gompertz model are summarized in Table 1. The statistical analysis gives a *p* value of less than 0.0002 and an r^2 value of higher than 0.98. The maximum hydrogen potential, 1,182 ml, was observed at pH 5. The hydrogen production potential and specific hydrogen production rate decreased with increasing pH values between 5.0 and 9.0. As expected from the lack of sucrose degradation (Fig. 3), hydrogen production was not observed at pH 3 and 10, indicating that no or negligible biological growth occurred at these pHs.

The maximum hydrogen yield and specific hydrogen production rate observed at pH 5 were 117.6 ml/g COD and 112.5 ml/g VSS-h, respectively. The maximum hydrogen yield of 1.71 mol H_2/mol sucrose found in this study is significantly higher than the maximum value of 1.09 mol H_2/mol sucrose reported by Hawkes *et al.* [11]. Based on the fact that 1 mol of sucrose produces 8 mol of H_2, the maximum theoretical hydrogen conversion ratio was 22.56%. Whereas the maximum hydrogen yield was obtained at pH 5, the maximum microbial growth was obtained at pH 7, demonstrating that bacterial growth was not proportional to hydrogen production. Microbial community shift likely occurred at different pH conditions, resulting in different bacterial growth rates.

Production of VFA and COD Balance

Hydrogen production is usually accompanied by acid production, which is, in turn, coupled with solvent production such as ethanol, butanol, and acetone. The production of these intermediates reflects changes in the metabolic pathways of the microorganisms involved [5]. Fig. 5 illustrates the distribution of the key VFAs (volatile fatty acids) in the reactor at various pHs. It shows that acetate and formate were the two most abundant species at pH values higher than 5. Increase of pH from 4.0 to 9.0 resulted in increases of acetate and formate formation and decreases of butyrate formation. Butyrate was not observed in the range of pH 7.0 to pH 10.0. Propionate was not detected in this study.

The ratio of butyrate to acetate was 1.63 at pH 5.0 and 0.38 at pH 6.0. These results might be interpreted in that the higher ratio at pH 5.0 could have driven the reaction predominantly towards higher production of hydrogen. The changes in the butyrate/acetate ratio implied a microbial community shift due to pH changes, resulting in lower production of hydrogen. This finding was in close agreement with previous results that acetogens consuming hydrogen would be predominant when the butyrate/acetate ratio becomes lower [14, 20].

In this study, more production of acetic acid and formic acid was observed at a higher pH, but the production of hydrogen was decreased. Das and Veziroglu [7] reported that hydrogen productions were decreased by the formation of formate [Eqs. (3) and (4)], being consistent with our observations (Fig 5). Less hydrogen productions with higher acetate concentrations in our study are consistent with microbial transformation of glucose into acetate (Eq.

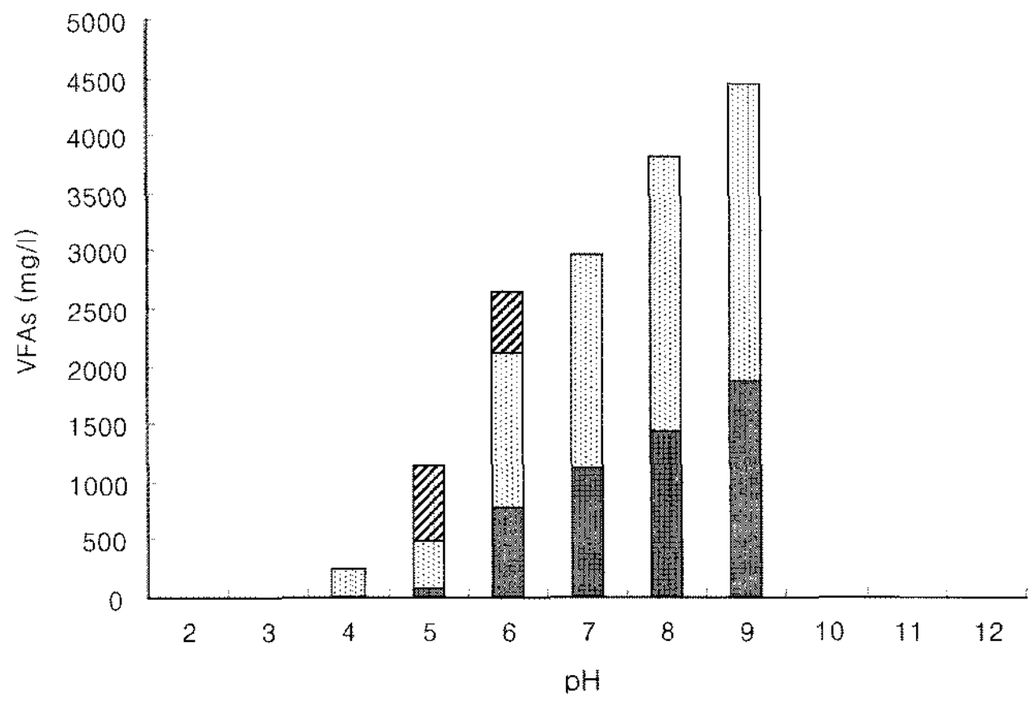


Fig. 5. Organic acid profiles under various pH condition. VFAs: volatile fatty acids.

Table 2. COD balance with varying pH (unit: %).

pH	Initial COD	VFAs			Biomass	Sucrose remaining	Others	H_2	Recovery
		Formate	Acetate	Butyrate					
3	100	–	–	–	–	96.71	3.29	–	100.00
4	100	–	2.36	–	1.76	63.85	19.50	4.32	91.79
5	100	0.24	4.06	11.29	7.62	0.48	68.54	7.98	100.22
6	100	2.56	13.53	8.85	8.39	0.97	58.71	6.34	99.35
7	100	3.84	19.41	–	7.83	2.01	62.74	4.18	92.01
8	100	6.28	24.07	–	8.13	0.97	53.29	1.42	94.16
9	100	6.04	24.25	–	5.64	0.45	58.55	0.15	95.08
10	100	–	–	–	–	92.74	3.93	–	96.67

Others: Soluble COD–(VFA+soluble carbohydrate)

5) and/or homoacetogenesis with hydrogen consumption (Eq. 6), as reported by Gavala *et al.* [9].

$$NADH+H^+ \rightarrow H_2+NAD^+ \quad (3)$$

$$CH_3COCOOH+CO_2+NADH+H^+ \rightarrow 2HCOOH+H_2O+NAD^+ \quad (4)$$

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \quad (5)$$

$$4H_2+2CO_2 \rightarrow CH_3COOH+2H_2O \quad (6)$$

Table 2 summarizes the overall COD balance. Initial COD was calculated from the sucrose concentration, whereas those of the fatty acids were measured from experiments. The carbon content in the biomass was calculated by assuming the composition of $C_5H_7O_2N$. Others refers to the solvent concentrations of products that were not detected by the UV detector on HPLC at 210 nm, such as ethanol or butanol. Table 2 shows that the overall COD balance was 91.8–100.2%. For pH values higher than 5, there was almost no difference in the biomasses although hydrogen production decreased. This demonstrates the microbial community shift in the batch kinetic reactor during the experiment.

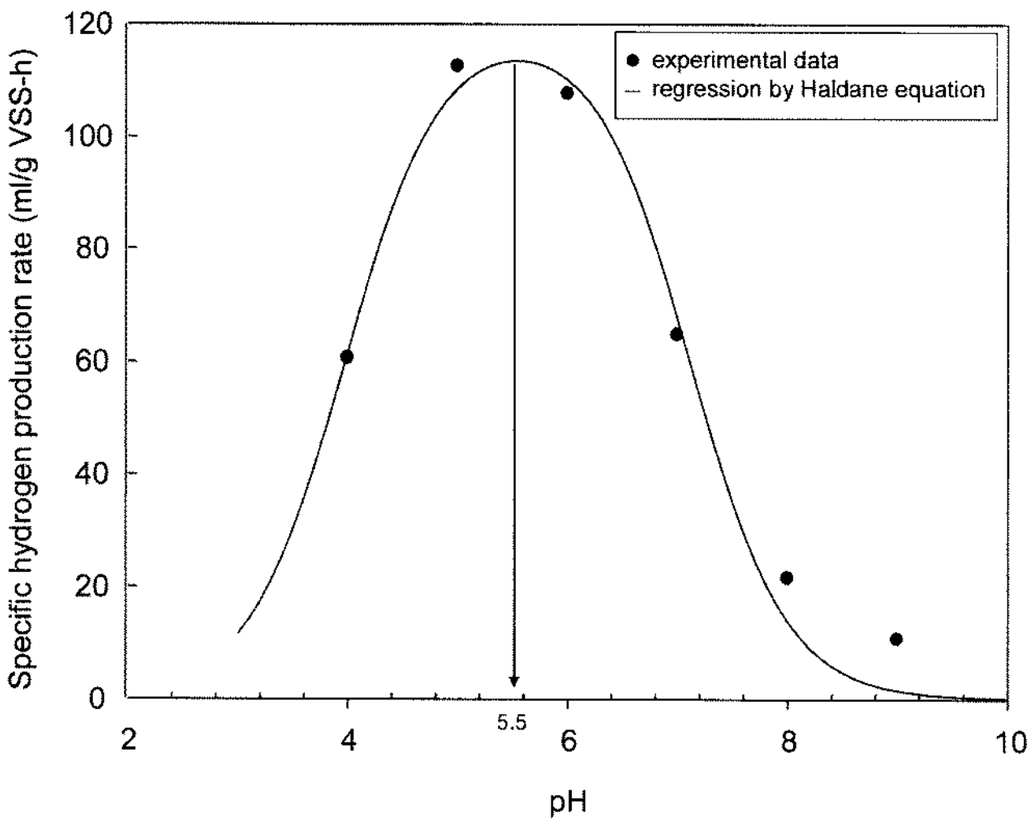


Fig. 6. Effect of pH on the specific hydrogen production rate by mixed microbial flora.
Regression of experimental data was conducted with Sigma Plot of SPSS Inc. (r^2=0.98; p-values of R_m, K_{OH}, and K_H were 0.0005, 0.0470, and 0.0496, respectively).

Kinetic Analysis of Hydrogen Ion Inhibition

The effect of pH on bacterial growth rate may be attributed to hydrogen ion in the medium. Tang *et al.* [30] reported that hydrogen ion inhibited the microbial fermentation following noncompetitive inhibition kinetics at pH values below 7.6. Stronger inhibition by acetic acid was found at a lower medium pH. The effect of hydrogen ion concentration on hydrogen production is described with the Haldane equation (Eq. 2).

The kinetic parameters obtained from fitting the specific hydrogen production rates in Table 1 with Eq. (2) were $K_H=1.07\times10^{-4}$ and $K_{OH}=7.69\times10^{-8}$. The maximum specific hydrogen production rate was 119.6 ml/g VSS-h. These parameters were used to simulate the model shown in Fig. 6. According to Eq. (2), the optimum pH of 5.5 for the specific hydrogen production rate is equal to $(pK_{OH}+pK_H)/2$. The model fits the experimental data well with the Haldane equation at all pH ranges (r^2=0.98).

In summary, it was found that pH changes in the reactor did have a significant effect on both sucrose degradation and hydrogen production. The cumulative hydrogen production in the batch experiment can be simulated well by a simple model developed from the Gompertz equation. The relationship between hydrogen ion concentrations and microbial activities was described well by a modified Haldane equation. The changes in the environmental conditions caused by varying pH values may have resulted in the alteration of the intermediate production pattern, especially the concentration ratios between volatile fatty acids.

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