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*Research Article*

# Biohydrogen Production from Cassava Starch Manufacturing Wastewater

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**Abstract.** Batch production of biohydrogen from cassava wastewater were investigated using (i) anaerobic seed sludge, (ii) a mixed culture of anaerobic seed sludge and *Rhodospirillum rubrum*, and (iii) a two-step batch culture of anaerobic seed sludge and *R. rubrum*. Maximum hydrogen production in batch culture of anaerobic seed sludge was achieved at 55 $^{o}$C and pH 5.0 with a specific hydrogen production of 429 mL $H_2$ $g^{-1}$-VSS and a hydrogen yield of 71.3 mL $g^{-1}$-COD. Results from hydrogen production by the mixed culture showed that the presence of *R. rubrum* improved the specific hydrogen production by 1.5-fold and the hydrogen yield by 2.1-fold when compared to the use of anaerobic seed sludge at the same conditions of 30$^{o}$C and pH 7.0. Superior results were obtained when the two-step batch culture, which involved the sequential addition of anaerobic seed sludge and *R. rubrum*, was used for hydrogen production. The cumulative hydrogen of 67 mL was produced at 30$^{o}$C and pH 7.0. Our results suggested that cassava wastewater is one of potential sources of renewable biomass to produce hydrogen.

**Keywords:** Hydrogen Production, Cassava Wastewater, Anaerobic Digestion, *Rhodospirillum Rubrum*.

## Introduction

Thailand produces cassava starch about 18 million tons per year. One kilogram of fresh roots yields 0.2 kg of starch, 0.4- 0.9 kg of cake and about 5-7 L of wastewater [1]. Cassava wastewater is a carbohydrate-rich waste. The wastewater contains high BOD, COD and Total Solids (TS) as organic substances are extracted from the cassava roots. The production of biogas from cassava starch wastewater has already been proven to be feasible but the information on hydrogen production from cassava starch manufacturing waste is very limited.

Hydrogen is now being received more attention as an alternative to fossil energy sources due to the fact that the combustion product of hydrogen is non-polluting chemical i.e., water and a high energy yield of 122 kJ $g^{-1}$ which is 2.75-fold of hydrocarbon fuels. Hydrogen can be generated in 4 ways i.e., (1) electrochemical processes; (2) thermochemical processes; (3) photochemical process, photocatalytic process, or photo electrochemical process and (4) microbial process. The first 3 processes have the disadvantages in which they do not reduce waste, do not produce energy and require electricity derived from fossil fuel combustion. On the other hand, microbial process produces energy and reduces waste [2]. Microbial hydrogen production can be classified into two categories (i) by photosynthetic microorganisms such as algae and photosynthetic bacteria; and (ii) by fermentative hydrogen-producing microorganisms such as facultative anaerobes and obligate anaerobes [3]. A system of combining anaerobic bacteria with photosynthetic bacteria can produce hydrogen from residual carbohydrates, for example, from organic wastes [4-8]. In such system, anaerobic fermentation of organic wastes produces organic acids as the intermediates, which are then converted into hydrogen by photosynthertic bacteria [9]. One of the photosynthetic bacteria that has been researched for its ability to produce hydrogen is *Rhodospirillum rubrum*. *R. rubrum* has shown the ability to use various kinds of substrate e.g., lactate [10], whey or yogurt [11]. In addition, it had shown the ability to use organic acids to produce hydrogen [12]. Not only production of hydrogen that *R. rubrum* is capable of but also the COD removal. *R. rubrum* was reported to remove about 22% COD from distillery waste [13].

Research in production of hydrogen from starch involved the use of pure culture strains such as *C. butyricum, Rhodobacter sp*. M-19, and *E. aerogenes*. A mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes* [14] and a mixed culture of *Clostridium butyricum* and *Rhodobacter sp*. M-19 [6] produced hydrogen from starch at a yield about 2 and 6.6 mol hydrogen/mol glucose, respectively. As of our knowledge, there is no information on using mixed cultures i.e., anaerobic mixed culture from anaerobic treatment pond and/or photosynthetic bacteria to produce hydrogen from starch waste. The advantages of using mixed cultures over pure culture including (i) lower cost (no sterile condition is needed); (ii) non-sterile organic wastes can be used as substrate; and (iii) possible to control operation base on differential kinetics of microbial subgroups [15]. Therefore, in this study we aimed to investigate a production of biohydrogen from cassava starch- manufacturing wastewater using (i) anaerobic seed sludge, (ii) a mixed culture of anaerobic seed sludge and *Rhodospirillum rubrum*, and (iii) a two-step batch culture of anaerobic seed sludge and *R. rubrum*. Information from this study will be useful in recovering bioenergy, hydrogen gas, from wastes derived from renewable resources.

## Material and Methods

### *Wastewater*

Cassava wastewater was obtained from Asia Citric Factory, Kalasin Province, Thailand. The pH value of this cassava wastewater was 5.02. Its COD, total nitrogen and total phosphorus values were 22,600, 258 and 54 mg $L^{-1}$, respectively.

### *Seed Inoculums*

Anaerobic seed sludge was obtained from sewage anaerobic sludge digestion plant in Ube City, Yamaguchi, Japan. The seed sludge was filtered through a screen (mesh size of 1,000 μm) and kept at 4°C prior to use. When used, 10 mL of anaerobic seed sludge was washed by centrifuging at 12,000 rpm for 10 min at 4°C and then re-suspended in 10 mL of sterile milli-Q water. Volatile Suspended Solid (VSS) and Total Suspended Solid (TSS) of seed inoculums were 13,300 and 19,300 mg $L^{-1}$, respectively, determined by standard method [16]. The seed sludge showed amylase activity by hydrolyzing starch using the method described previously [14]. *R. rubrum, DSM 467,* was cultivated anaerobically under illumination of 6,000 lux using fluorescent lamp at 30°C for 7 days in growth medium (pH 7.2) consisting of 1% $K_2HPO_4$, 0.5% $MgSO_4$, 10% yeast extract, 2.01% DL-malic acid and 0.33% DL-glutamic acid.

### *Hydrogen production by anaerobic seed sludge*

This experiment was conducted to investigate the ability of microorganisms in anaerobic seed sludge to produce hydrogen from cassava wastewater at various initial pH (5, 6 and 7) and cultured temperature i.e. mesophilic (30 and 35 °C) and thermophilic (45 and 55°C). The series of batch experiment were conducted in 75 mL glass serum bottle with a working volume of 50 mL. Cassava wastewater was adjusted to have COD:N:P at COD 20,000 mg $L^{-1}$ of 100:10:1 mg $L^{-1}$ by using NH4Cl and K2HPO4 as N-source and P-source, respectively. Ten mL of anaerobic seed sludge and 40 mL of cassava wastewater were added to serum bottles and the initial pH of liquid contents was adjusted to 5, 6, 7 by adding 1N HCl or 1N NaOH. The gas phase was then replaced with argon to create anaerobic condition. The batch experiment was then cultured in the water bath at two selected conditions i.e. mesophilic condition (30 and 35 °C) and thermophilic condition (45 and 55 °C).

### *Hydrogen production by a mixed culture of anaerobic seed sludge and R. rubrum*

This experiment was designed to examine the hydrogen production system using the co-culture of anaerobic seed sludge and *R. rubrum*. Therefore, 10 mL of anaerobic seed sludge, 10 mL of the cell suspension of *R. rubrum* and 30 mL of the cassava wastewater at 20,000 mg $L^{-1}$ (COD:N:P of 100:10:1) were put in the 75 mL serum bottle. The initial pH of liquid contents was adjusted to different values i.e., 5, 6, 7 by adding 1 N HCl or 1 N NaOH. After the bottles were flushed with argon to replace the gas phase, the cells were cultured at 30°C without a pH control under illumination at 6000 lx by the fluorescent lamp.

### *Hydrogen production by a two-step system of anaerobic seed sludge and R. rubrum*

In this system we intended to enhance a production of hydrogen from cassava wastewater by sequentially addition of anaerobic seed sludge and *R. rubrum*. After 110 hrs of culturing anaerobic seed sludge in cassava wastewater at 0,000 mg $L^{-1}$ (COD:N:P of 100:10:1) with various initial pH of 5, 6 and 7, the anaerobic seed sludge was filtered out
from the culture broth through the glass fiber filter, size of 47 mm (Toyo Roshi Kaisha,

Ltd.).Then, 10 mL of cell suspension of *R. rubrum* was added to the remaining 40 mL of the culture broth in the serum bottle and the pH was adjusted to 7 by adding 1N NaOH or 1 N HCl. The gas phase was then replaced with argon and the serum bottles were incubated at 30$^{o}$C without a pH control under illumination at 6000 lx by the fluorescent lamp.

**Analytical method**

***Gas Analysis***

The volume of biogas was measured by plunger displacement method using appropriately sized wetted glass syringes following the method presented by Owen et al.[17]. The components of biogas were analyzed by a gas chromatograph (GC) (Model 8APT, SHIMADZU, Japan) equipped with a thermal conductivity detector (TCD). A 3m x 3mm diameter stainless-steel column packed with activated charcoal (60/80 mesh) (Model 8APF, SHIMADZU, Japan) was used to analyze the percentage of hydrogen, nitrogen, methane and carbon dioxide in biogas produced. Argon was used as the carrier gas at a flow rate of 70 mL min$^{-1}$. The temperatures of injector, detector and column were 50, 50 and 60$^{o}$C, respectively.

***Volatile Fatty Acids (VFAs) Analysis***

The concentration of VFAs, including acetic, propionic, and n-butyric were determined by a gas chromatograph (Model 8A, SHIMADZU, Japan) equipped with a flame ionization detector (FID) and a 3m x 3.2 mm diameter glass column packed Unisole F-200 (30/60 mesh) (GL Science Inc. Japan). Injector, detector and column temperatures used were at 250, 140 and 140$^{o}$C, respectively. Nitrogen, hydrogen and pressured air were used as carrier gases with flow rate of 50, 60 and 500 mL min$^{-1}$, respectively.

***Kinetic analysis***

The cumulative hydrogen production in the anaerobic batch experiments using seed sludge followed the modified Gompertz equation (Equation 1).

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

*H (t)* is the cumulative volume of hydrogen produced (mL), *P* is the hydrogen production potential (mL), $R_m$ is the maximum production rate (mL h$^{-1}$), e is 2.71828 and λ is the lag time (hr) and t is time. The maximum specific hydrogen production rate (mLg$^{-1}$-VSS.hr) was calculated by dividing $R_m$ by the initial sludge VSS. The hydrogen yield or conversion efficient (mL g$^{-1}$-COD wastewater) was calculated by dividing P by the g-COD waste water. The specific hydrogen production (mL $H_2$ g$^{-1}$-VSS) was calculated by dividing P by g-VSS.

**Results and Discussion**

***Hydrogen production from cassava wastewater by anaerobic seed sludge***

Mesophilic temperature (30 and 35$^{o}$C) and thermophilic temperature (45 and 55$^{o}$C) were used to culture anaerobic seed sludge to produce hydrogen from cassava wastewater with the initial pH of 5, 6 and 7. The results showed that the main organic acid produced at mesophilic temperature (30 and 35$^{o}$C) was acetic acid (HAc) (Table 1) suggesting that the reaction was

a HAc fermentation type. Normally fermentation products of acidogenic fermentation are butyric acid (HBu) [18]. However, different fermentation products could be obtained if the culture conditions of the bacteria groups contributing to the fermentation process are changed [19-22]. The results also indicated that HAc was produced at pH 5>pH 6>pH 7 at mesophilic temperature (30 and 35 $^{o}$C) (Table 1). This can be explained by Equation (2) that at a lower initial pH the reaction drives toward acetate production resulting in high HAc produced.

$$C_{12}H_{22}O_{11}+5H_2O \longrightarrow 4CH_3COOH+4CO_2+8H_2 \quad (2)$$

At thermophilic temperature (45 and 55$^{o}$C) the HAc and HBu were major components (about 50% for each) except at pH 5, 55$^{o}$C, 94% of HBu was obtained. *Clostridium* species might be responsible for this trend since *Clostridium* species are known for butyrate fermentation [23-24]. Lin and Chang [25] stated that *Clostridium* ferment sugars and starch and the products obtained are butyrate, acetate, $CO_2$ and $O_2$. *C aceticum* can reduce $CO_2$ to acetate with $H_2$ as an electron donor. Previous research demonstrated that *C. acetobutylicum* did not produce propionic acid (HPr) in its metabolic pathway which is in agreement with our results as showed in Table 1 in which a very small amount of HPr was generated in our experiments. A little percentage of HPr produced may be due to the fact that our experiment used the mixed cultures but the previous reports used the pure strain. Chen et al. [26] explained that the microorganisms in mixed culture had some symbiotic nature or syntrophic interactions that produced HPr to support our speculation that *Clostridium* species is the dominant microorganism at thermophilic temperature (45 and 55$^{o}$C). We tested the amylase activity of the *Clostridium* species in anaerobic seed sludge by hydrolyzing the starch and found that the anaerobic seed sludge had amylase activity of $8.5x10^{7}$ CFUmL$^{-1}$. According to Valdez-Vazquez et al. [15] which stated that in the anaerobic mixed cultures, bacteria of *Clostridia* genera are present in great proportion. Due to above reasons we speculated that the dominant microorganism in our anaerobic seed sludge at thermophilic temperature was from *Clostridia* genera. More investigation on identifying the microorganisms using microbiology method such as API system should be conducted.

**Table 1** Effect of initial pH of cassava wastewater and cultivation temperature on production of VFAs. The data are given as mean±SD, n = 2

| Initial pH | Temp ($^{o}$C) | Final pH | HAc (%) | HPr (%) | HBu (%) | HBu/HAc (B/A) ratio |
|---|---|---|---|---|---|---|
| 5 | 30 | 3.5±0.9 | 93.8±1.9 | 1.9±0.1 | 4.2±1.9 | 0.05 |
| | 35 | 4.3±0.2 | 95.9±1.3 | 1.8±0.2 | 2.1±1.0 | 0.02 |
| | 45 | 3.5±0.2 | 56.3±12.1 | 0.4±0.1 | 43.25.5 | 0.77 |
| | 55 | 4.6±0.2 | 5.2±1.2 | 0.3±0.2 | 94.3±9.6 | 18.01 |
| 6 | 30 | 4.4±0.3 | 79.1±3.7 | 4.3±1.4 | 16.4±5.2 | 0.21 |
| | 35 | 3.6±0.1 | 96.6±0.1 | 1.9±0.1 | 1.3±0.0 | 0.01 |
| | 45 | 3.4±0.1 | 50.8±4.2 | 0.7±0.2 | 48.4±0.5 | 0.95 |
| | 55 | 4.9±0.1 | 46.5±0.1 | 2.6±0.1 | 50.8±0.0 | 1.09 |
| 7 | 30 | 4.9±0.0 | 73.2±0.8 | 2.6±0.5 | 24.1±0.2 | 0.33 |
| | 35 | 3.8±0.2 | 74.8±0.2 | 3.9±0.2 | 21.1±0.4 | 0.28 |
| | 45 | 3.4±0.2 | 50.7±2.2 | 0.3±0.0 | 48.9±2.3 | 0.96 |
| | 55 | 5.2±0.4 | 68.3±0.1 | 4.2±0.3 | 27.4±0.6 | 0.40 |

Final pH dropped below 5 at each experimental condition (Table 1). This may result from the

VFAs produced in each serum bottles. The indicator for evaluating the effectiveness of $H_2$ production normally used is HBu/HAc ratio. The results revealed that for higher HBu/HAc ratios (Table 1), a higher maximum specific hydrogen production rate was obtained (Table 2). Different in the anaerobic cultures and the substrate used resulted in different optimal HBu/HAc ratio for $H_2$ production [27]. For instance, the optimal HBu/HAc ratios for *C. butyricum* and *Butyribacterium methylotrophicum* were reported to be 2 and 0.75, respectively. In our experiment, we found that the maximum HBu/HAc for production of $H_2$ from cassava wastewater by anaerobic seed sludge was 18.01 at pH 5, 55$^{o}$C (Table 1).

This result correspond to the maximum hydrogen production potential (P), the highest hydrogen yield, and the highest specific hydrogen production of 58.5 mL, 71 mL $g^{-1}$-COD and 440 mL $H_2$ $g^{-1}$-VSS, respectively (Table 2). Therefore, we concluded that the optimum condition for hydrogen production from cassava wastewater by anaerobic seed sludge was pH 5 and 55$^{o}$C. The data also indicated that thermophilic temperature was more suitable to produce hydrogen from cassava wastewater by anaerobic seed sludge than mesophilic temperature (Table 2).

We observed that at thermophilic temperature, 45 and 55 $^{o}$C, when the P values were high the $R_m$ values were also high. Chen et al. [26] explained that this may due to the fact that sludge microorganisms posses a range of responsive capacities for different adverse circumstances resulted in a certain characteristic, such as P, $R_m$ and λ. The λ value was high at 55$^{o}$C at all initial pH. This indicated that sludge microorganisms needed a longer lag phase time to adjust to a new environment at high temperature. However, results from hydrogen production by the mixed culture showed that the presence of *R. rubrum* improved the specific hydrogen production by 1.5-fold and the hydrogen yield by 2.1-fold (Table 3) when compared to the use of anaerobic seed sludge at the same conditions of 30$^{o}$C and pH 7.0 (Table 2). This indicated that *R.rubrum* was effective in producing hydrogen.

***Hydrogen production from cassava wastewater by a mixed culture of anaerobic seed sludge and R. rubrum***

This experiment examined the ability of anaerobic seed sludge and *R. rubrum* to co-produce hydrogen from cassava wastewater in a single step system at different initial pH and a mesophilic temperature of 30$^{o}$C. Fig. 1 depicted that HBu was the major intermediate organic acid at each initial pH suggesting a butyrate fermentation mode. The highest percentage of HBu (Fig. 1) and the maximum P value were obtained at the initial pH 7 (Table 3) indicating that volume of hydrogen gas produced depended on the concentration of butyric acid. However, we observed that the hydrogen gas volume produced by this single step were very low at all pH levels i.e., P values of 4.5, 9.0 and 12.5 mL at the initial pH of 5, 6 and 7, respectively (Table 3). This may due to the fact that $H_2$ produced was consumed by hydrogen consuming bacteria present in the system. In anaerobic digestion plant, a consortium of microorganisms converts organic waste into a mixture of $CH_4$ and $CO_2$. Prior to the methanogenic stage, hydrogen was obtained as an intermediate product and was utilized as it was produced by methanogenic archea, acetogenic bacteria and sulfate reducing bacteria [15]. Therefore, if the hydrogen consuming bacteria can be inhibited, the more hydrogen produced will be obtained. The other reason that the volume of hydrogen evolved was low (Fig.2) may due to the fact that *R. rubrum* could not outcompete natural microorganisms in anaerobic sludge. The inhibition of hydrogen consuming microorganisms can be done by using low pH, heat-shock pretreatment and using chemical compounds such as chloroform, fluoroacetate, and acetylene [15]

**Table 2** Modified Gompertz equation parameters for hydrogen production from cassava wastewater, 10,000 $mgl^{-1}$, by anaerobic seed sludge

| Initial pH | T (°C) | λ (h) | $R_m$ (mL $h^{-1}$) | P (mL) | Maximum specific $H_2$ production rate (mL $g^{-1}$-VSS-h) | Specific hydrogen production (mL $H_2$ $g^{-1}$-VSS) | Hydrogen yield (mL $g^{-1}$ COD) | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| 5 | 30 | 2.4 | 0.03 | 0.3 | 0.0 | 2.0 | 0.4 | 0.98 |
| | 35 | 2.5 | 0.02 | 0.4 | 0.2 | 2.0 | 0.4 | 0.95 |
| | 45 | 4.0 | 3.50 | 31.0 | 26.0 | 233.0 | 39.0 | 0.99 |
| | 55 | 103.0 | 1.20 | 58.5 | 9.0 | 440.0 | 71.0 | 0.96 |
| 6 | 30 | 3.0 | 0.07 | 0.5 | 0.5 | 3.8 | 0.6 | 0.99 |
| | 35 | 2.0 | 0.15 | 1.6 | 1.0 | 12.0 | 2.0 | 0.90 |
| | 45 | 4.5 | 5.00 | 32.0 | 37.6 | 240.6 | 40.0 | 0.91 |
| | 55 | 9.5 | 1.00 | 13.0 | 7.5 | 98.0 | 16.0 | 0.99 |
| 7 | 30 | 5.0 | 0.50 | 8.0 | 3.8 | 60.0 | 10.0 | 0.99 |
| | 35 | 1.2 | 1.80 | 22.5 | 13.5 | 169.0 | 28.0 | 0.98 |
| | 45 | 3.0 | 1.00 | 45.0 | 7.5 | 338.0 | 56.0 | 0.99 |
| | 55 | 28.0 | 0.20 | 6.5 | 1.5 | 49.0 | 8.0 | 0.97 |

***Hydrogen production from cassava wastewater by a two-step batch culture of anaerobic seed sludge and R. rubrum***

In this system we intended to enhance a production of hydrogen from cassava wastewater by letting

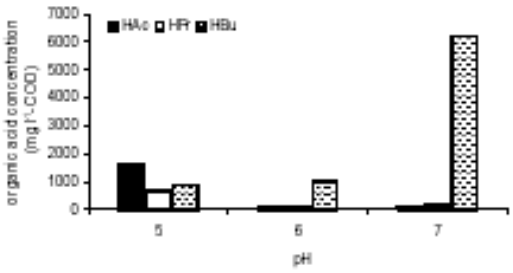


**Fig. 1** The contents of organic acids produced as the intermediates by a mixed culture of anaerobic seed sludge and *R. rubrum*.

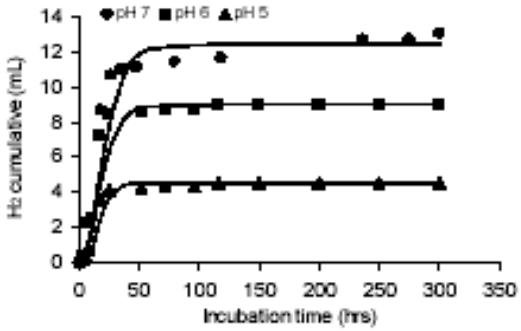


**Fig. 2** Hydrogen cumulative by mixed culture of anaerobic seed sludge and *R. rubrum.*

Anaerobic seed sludge converted starch in cassava wastewater to organic acids in the first step and then in the second step *R. rubrum* produced hydrogen using

**Table 3** Modified Gompertz equation parameters for hydrogen production from assava wastewater, 10,000 mg $L^{-1}$, by a mixed culture of anaerobic seed sludge and *R.rubrum*.

| Initial pH | Temp ($^0$C) | λ (hr) | $R_m$ (mL $hr^{-1}$) | *P* (mL) | Maximum specific $H_2$ production rate (mL $g^{-1}$-VSS-d) | Specific hydrogen production (mL $H_2$ $g^{-1}$-VSS) | Hydrogen yield (mL $g^{-1}$ COD) | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| 5 | 30 | 2 | 0.24 | 4.5 | 1.8 | 43.0 | 0.2 | 0.99 |
| 6 | 30 | 2 | 0.40 | 9.0 | 2.9 | 76.0 | 0.5 | 0.99 |
| 7 | 30 | 2 | 0.60 | 12.5 | 4.4 | 105.0 | 0.6 | 0.99 |

organic acids obtained in culture broth. We adjusted the pH of the culture broth from below 5 to pH 7 before adding *R. rubrum* into the culture broth. This was because there was no $H_2$ gas produced without pH adjusted (data not shown) suggesting *R. rubrum* cannot grow at low pH and the optimum pH of *R. rubrum* was pH7. Fig. 3 showed that the main intermediates were HAc, HBu and HPr. Concentration of HBu was maximum at the initial pH of 7 (Fig. 3) and at this pH the maximum of hydrogen gas evolved i.e, 67 mL (Fig. 4) was obtained indicating a sequential addition of anaerobic seed sludge and *R. rubrum* consequence in superior results of hydrogen production.

## Conclusions

In this research we have found that: 1) Maximum hydrogen production in batch culture of anaerobic seed sludge was achieved at 55 oC and pH 5.0 with a specific hydrogen production of 440 mL H2g-1-VSS and a hydrogen yield of 71 mLg-1-COD. 2) Results from hydrogen production by the mixed culture showed that the presence of *R. rubrum* improved the specific hydrogen production by 1.5-fold and the hydrogen yield by 2.1-fold when compared to the use of anaerobic seed sludge at the same conditions of 30oC and pH 7.0.

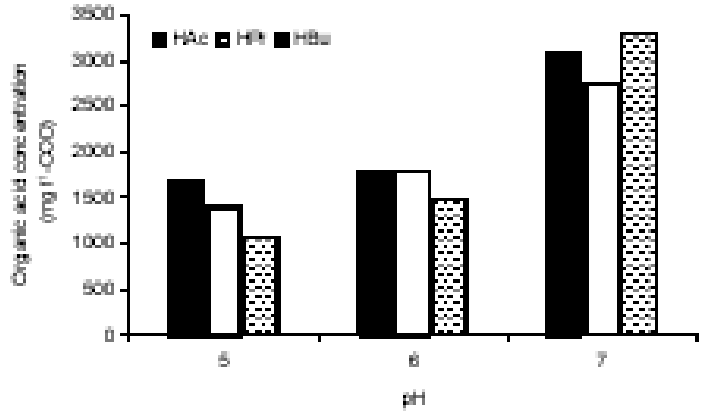


**Fig. 3** Concentration of organic acids produced by a two-step batch culture of anaerobic seed sludge and *R. rubrum*

3) Superior results were obtained when the two-step batch culture, which involved the sequential addition of anaerobic seed sludge and *R. rubrum*, was used for hydrogen

production. The cumulative hydrogen of 67 mL was produced at 30$^{O}$C and pH 7.0

4) Our results suggested that cassava wastewater isone of potential sources of renewable biomass to produce hydrogen.

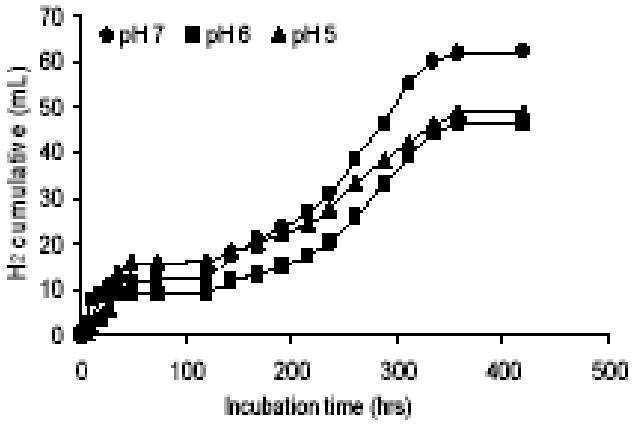


**Fig. 4** Hydrogen cumulative by a two-step batch culture of anaerobic seed sludge and *R. rubrum*.

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