



รายงานวิจัยฉบับสมบูรณ์

โครงการ: การผลิตไฮโดรเจนจากน้ำทิ้งโรงงานผลิตแอมโมเนียสำหรับผลิตแอมโมเนียมซัลเฟต  
Upflow Anaerobic Sludge Blanket

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สนับสนุนโดยทบวงมหาวิทยาลัย และสำนักงานกองทุนสนับสนุนการวิจัย  
(ความเห็นในรายงานนี้เป็นของผู้วิจัย ทบวงฯ และ สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

## ABSTRACT

This research investigated the feasibility of using cassava starch manufacturing wastewater as a substrate for hydrogen production in anaerobic fermentation process. A series of batch tests were conducted to investigate the bihydrogen production potential from cassava starch manufacturing wastewater at 20,000 mg-COD/L using three different inocula, namely (i) anaerobic sludge, (ii) co-culture of anaerobic sludge and *Rhodospirillum rubrum* and (iii) a sequential addition of anaerobic sludge and *R. rubrum*. Using anaerobic sludge alone, the maximum specific hydrogen production of 1,511 mL H<sub>2</sub>/g-VSS and the maximum hydrogen yield of 251 mL/g-COD fed were obtained at thermophilic temperature (55°C) and initial pH 5.0. The use of co-culture of anaerobic sludge and *R. rubrum* in single stage as inoculum improved the specific hydrogen production by 1.7-fold and the hydrogen yield by 1.7-fold in comparison to the use of anaerobic sludge alone at the same test conditions of 30°C and initial pH 7.0. Superior results were obtained when a sequential addition of anaerobic sludge and *R. rubrum* was used for hydrogen production. The cumulative hydrogen of 300 mL with COD-H<sub>2</sub>/COD<sub>input</sub> of 0.28 was produced at 30°C and initial pH 7.0. This study indicated that cassava starch manufacturing wastewater has a potential for sustainable hydrogen production.

Sustainable hydrogen production from cassava wastewater was conducted in Upflow Anaerobic Sludge Blanket (UASB) reactor. Inoculum used to form UASB granule was anaerobic sludge obtained from the alcoholic wastewater treatment plant. Prior to granule formation, anaerobic sludge was heat treated in boiling water for 30 minutes. A pH of the substrate in the UASB reactor was maintained at pH 6-6.5 by adding NaHCO<sub>3</sub>. Heat shock treatment and pH control were conducted to inhibit methanogenic activity. The 9.4-L UASB reactor was operated for 7 months at 5 different hydraulic retention time (HRT) i.e., 24, 18, 12, 8.4 and 4.8 h with concentrations of cassava wastewater and inoculum approximately 10,000 mg COD/L and 10,000 mg/L, respectively. The biomass concentration in the bed zone of the UASB reactor was increased throughout the experiment suggesting that hydrogen producing granulated sludge were developed. A shift in HRT from 24 h to 12 h appeared to enhance hydrogen production rate. When HRT was shortened from 12 h to 4.8 h the percentage of hydrogen produced decreased from 46% to 10%. Peak of the hydrogen yield of 46 mL H<sub>2</sub>/g COD and the hydrogen production rate of 16.1 L/d were obtained at HRT 12

h. These results indicated that HRT 12 h was an optimum HRT in producing hydrogen from cassava wastewater. An average granular sludge at HRT 12 h was a light-grey in color and was 0.14 mm in diameter. Each gram of biomass produced 0.89 mL H<sub>2</sub>/day with gas evolved mixture of 46% hydrogen, 40% carbon dioxide and less than 2% methane. The effluent volatile suspended solid, the endogenous decay coefficient (K<sub>d</sub>) and yield coefficient (Y<sub>g</sub>) of hydrogen producer granules were 235 mg/L and 0.64 /day and 0.93 g VSS/g COD, respectively. During the efficient hydrogen production stage, a major soluble metabolite was butyric acid, followed by acetic acid and propionic acid. Analysis of microbial by DGGE in granulated sludge indicated that dominant species found in each band on the DGGE profile at every HRT were *Megasphaera elsdenii* and *Megasphaera honisis*.

**Keywords:** anaerobic sludge; biohydrogen production; cassava starch manufacturing wastewater; *Rhodospirillum rubrum*, microbial community, UASB

## บทคัดย่อ

งานวิจัยนี้ได้ศึกษาความเป็นไปได้ในการผลิตไฮโดรเจน โดยใช้น้ำทิ้งโรงงานแป้งมันเป็นสับเสตรท ในกระบวนการหมักแบบไร้อากาศ การทดลองแบ่งออกเป็น 2 ส่วน ส่วนแรก เป็นการทดลองหมักแบบกะ โดยใช้น้ำทิ้งโรงงานแป้งมันสำปะหลังที่มีความเข้มข้น 20,000 มิลลิกรัม COD ต่อลิตร เป็นสับเสตรท โดยใช้เชื้อจุลินทรีย์ที่แตกต่างกัน 3 ชนิด คือ 1) เชื้อจุลินทรีย์ที่ได้จากกากตะกอนจากระบบบำบัดน้ำเสียแบบไร้อากาศ (anaerobic sludge) 2) เชื้อจุลินทรีย์สองชนิดร่วมกัน ได้แก่ anaerobic sludge และ *Rhodospirillum rubrum* และ 3) ทำการหมักโดยใช้ anaerobic sludge ก่อน แล้วหมักต่อด้วย *R. rubrum* ผลการทดลองพบว่า การหมักโดยใช้ anaerobic sludge เพียงอย่างเดียว ที่อุณหภูมิสูง (50 องศาเซลเซียส) และค่าพีเอชเริ่มต้นเท่ากับ 5.0 ให้ค่าการผลิตไฮโดรเจนจำเพาะสูงสุด (maximum specific hydrogen production) เท่ากับ 1,1511 มิลลิกรัมไฮโดรเจนต่อกรัมสารแขวนลอยระเหยได้ (VSS) และผลได้ไฮโดรเจนสูงสุด (maximum hydrogen yield) เท่ากับ 251 มิลลิลิตรต่อกรัม COD เมื่อหมักโดยใช้ anaerobic sludge ร่วมกับ *R. rubrum* ที่อุณหภูมิ 30 องศาเซลเซียส และพีเอชเริ่มต้นเท่ากับ 7 พบว่าค่าการผลิตไฮโดรเจนจำเพาะสูงสุดและค่าผลได้ไฮโดรเจนสูงสุดเพิ่มขึ้น 1.7 เท่า เมื่อเปรียบเทียบกับ การหมักโดยใช้ anaerobic sludge เพียงอย่างเดียว และการทดลองให้ผลดียิ่งขึ้นเมื่อทำการหมักโดยใช้ anaerobic sludge ก่อนแล้วจึงทำการหมักต่อโดยใช้ *R. rubrum* ที่อุณหภูมิ 30 องศาเซลเซียส พีเอชเริ่มต้นเท่ากับ 7 โดยให้ค่าไฮโดรเจนสะสมเมื่อสิ้นสุดการหมักเท่ากับ 30 มิลลิลิตร และค่าผลได้ไฮโดรเจนเท่ากับ 0.28 COD-ไฮโดรเจน ต่อ COD ที่เติมเข้าไปในระบบ จากผลการทดลองในส่วนนี้แสดงให้เห็นว่า ความเป็นไปได้ในการใช้น้ำทิ้งโรงงานแป้งมันสำปะหลังเพื่อเป็นสับเสตรทในการผลิตไฮโดรเจน

การทดลองในส่วนที่สองเป็นการผลิตไฮโดรเจนในถังหมักระบบ Upflow Anaerobic Sludge Blanket (UASB) เชื้อจุลินทรีย์ที่นำมาใช้ในการสร้างแกนรูสำหรับผลิตไฮโดรเจนในระบบ UASB นี้ เป็น anaerobic sludge จากระบบบำบัดน้ำเสียอุตสาหกรรมผลิตแอลกอฮอล์ โดยนำ anaerobic sludge ดังกล่าวมาต้มให้เดือดนาน 30 นาที ก่อนที่จะนำไปสร้างแกนรูในระบบ และทำการทดลองโดยควบคุมพีเอชของสับเสตรทในระบบให้อยู่ในช่วง 6.0-6.5 ซึ่งการทรिटด้วยความร้อนและควบคุมพีเอชที่ระดับนี้ทำเพื่อยับยั้งการเจริญและกิจกรรมของจุลินทรีย์ที่มีความสามารถในการผลิตมีเทน ทำการทดลองผลิตไฮโดรเจนในถังหมักระบบ UASB ขนาด 9.4 ลิตร นาน 7 เดือน แปรผันระยะเวลาการกักเก็บ (HRT) เป็น 24 18 12 8.4 และ 4.8 ชั่วโมง ความเข้มข้นของกากตะกอนเริ่มต้นในระบบเท่ากับ 10,000 มิลลิกรัมต่อลิตร และ ความเข้มข้นของน้ำทิ้งโรงงานแป้งมันที่เข้าสู่ระบบเท่ากับ 10,000 มิลลิกรัม COD ต่อลิตร ผลการทดลองพบว่าความเข้มข้นของชีวมวล (biomass) ในบริเวณ bed zone ของถังหมักมีการเพิ่มขึ้นตลอด

ระยะเวลาการทดลอง ซึ่งแสดงให้เห็นว่า มีการเพิ่มขึ้นของแกรนูลของเชื้อจุลินทรีย์ที่มีความสามารถในการผลิตไฮโดรเจน อัตราการผลิตไฮโดรเจนในระบบจะเพิ่มขึ้นเมื่อลด HRT จาก 24 ชั่วโมงเป็น 12 ชั่วโมง แต่อย่างไรก็ตาม การลด HRT จาก 12 ชั่วโมงเป็น 4.8 ชั่วโมง มีผลทำให้ความเข้มข้นของไฮโดรเจนที่ผลิตลดลงจาก 48 เปอร์เซ็นต์ ไปเป็น 10 เปอร์เซ็นต์ ผลได้ของไฮโดรเจนสูงสุด และอัตราการผลิตไฮโดรเจนสูงสุดที่ได้จากระบบ UASB นี้ มีค่าเท่ากับ 46 มิลลิลิตรไฮโดรเจนต่อกรัม COD และ 16.1 ลิตรต่อวัน ตามลำดับที่ HRT เท่ากับ 12 ชั่วโมง ผลการทดลองนี้แสดงให้เห็นว่า HRT ที่เหมาะสมที่สุดในการผลิตไฮโดรเจนจากน้ำทิ้งโรงงานแป้งมันสำปะหลัง คือ HRT 12 ชั่วโมง แกรนูลที่ได้จากระบบที่ HRT 12 ชั่วโมง มีลักษณะเป็นสีเทา มีขนาดเส้นผ่านศูนย์กลางเท่ากับ 0.14 มิลลิเมตร ปริมาณไฮโดรเจนที่ผลิตได้ต่อกรัมชีวมวลเท่ากับ 0.89 มิลลิลิตรไฮโดรเจนต่อวัน โดยแก๊สที่ผลิตได้ประกอบด้วย ไฮโดรเจน 46 เปอร์เซ็นต์ คาร์บอนไดออกไซด์ 40 เปอร์เซ็นต์ และมีเทนน้อยกว่า 2 เปอร์เซ็นต์ สารตัวกลางที่ตรวจพบในน้ำหมักระหว่างทำการผลิตไฮโดรเจนคือ กรดบิวไทริก กรดอะซิติก และกรดโพรไพโอนิก และผลการวิเคราะห์กลุ่มของจุลินทรีย์ในแกรนูลจากระบบด้วยเทคนิค Dentured Gradient Gel Electrophoresis พบว่ามีเชื้อจุลินทรีย์ที่เด่นสองชนิด คือ *Megasphaera elsdenii* และ *Megasphaera honisis* โดยพบเชื้อจุลินทรีย์ทั้งสองชนิดนี้ในแกรนูลที่ได้จากระบบทุก HRT

คำสำคัญ anaerobic sludge; biohydrogen production; cassava starch manufacturing

wastewater; *Rhodospirillum rubrum*, microbial community, UASB

## EXECUTIVE SUMMARY

### 1. Rational

Cassava starch production is a great extended industry in Thailand. The process to obtain starch from cassava root pollutes a huge volume of wastewater with high Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) which cause much environmental pollution (Richard and David, 2004). In addition, cassava wastewater contains intermediates for producing hydrogen i.e., acetic, propionic, and butyric acids of 415.78, 565.95 and 863.50 mg/L, respectively. (Polprasert, Rukvichitkul, 2003) In these instances, cassava wastewater can be a good candidate for bio-hydrogen production. Therefore, it is of our interest to recover energy from high organic content in the cassava wastewater before discharging it to the environment.

Biological hydrogen production through fermentation represents a new area of bioenergy production that could substitute non-renewable fossil fuels. It has many inherent merits including availability of naturally occurring microbes, a shorter hydraulic retention time (HRT), a simple bioreactor system, and potential for waste remediation. Biohydrogen production can be classified into 5 processes which are direct biophotolysis, indirect biophotolysis, biological water-gas shift reaction, dark fermentation and photo-fermentation (Levin et al., 2004). Among these 5 processes, microbial hydrogen production through fermentation receives much attention in hydrogen production in recent years due to its feasibility (Ni et al., 2005). Microbial hydrogen production is classified into two categories (Yokoi et al., 1995). One is hydrogen production by photosynthetic microorganisms such as algae or photosynthetic bacteria. The other is hydrogen production by fermentative hydrogen-producing microorganisms such as facultative anaerobes and obligate anaerobes (Yokoi et al., 1995). In general, the amount of hydrogen produced by dark fermentation is lower than that of photo-fermentation and the production rate is slower (Koku et al., 2003; Oh et al., 2004). However, dark fermentation has advantages over photo-fermentation including independence of solar radiation, less space requirement and independence of weather condition (Ni et al., 2005).

Sequential dark and photo-fermentation and combined dark and photo-fermentations for hydrogen production are new approaches in bio-hydrogen production. In such system,

anaerobic fermentation of organic wastes produces organic acids such as acetic and butyric acids which are the intermediates for hydrogen production by photosynthetic bacteria (Barbosa et al., 2001). Advantages of sequential dark and photo-fermentation system over single stage dark or photo-fermentation processes include (i) sufficient availability of organic acids from dark fermentation and (ii) better effluent quality due to the use of organic acids by photo-fermentative bacteria (Kapdan, Kargi, 2006). Sequential dark-photo fermentation technique was successfully used to produce hydrogen from residual carbohydrates such as sweet potato starch residue (Yokoi et al., 2001; Khanal et al., 2004); algal biomass (Kawaguchi et al., 2001) and solid waste (Fascetti et al., 1998).

Anaerobic dark continuous hydrogen production is the one effective technique because large volume of hydrogen could be produced continuously for a long period of operation time without inoculum preparation step (Chang, Lin, 2004). Upflow Anaerobic Sludge Blanket (UASB) process has been widely applied to the treatment of industrial wastewater with high organic concentration such as food and beverage industries (Lettinga et al., 1997; Lettinga et al., 1980). It is an extensively applied in continuous anaerobic treatment system with the advantage that high COD or BOD removal efficiency could be obtained at the short Hydraulic Retention Time (HRT) (Chang, Lin, 2004). Hydrogen production from various kinds of substrate by UASB reactor had been reported (Chang, Lin, 2004; Han, Shin, 2004; Yang et al., 2006). However, there is no information available on production of hydrogen from cassava wastewater by UASB process.

In the present study, a photosynthetic bacteria *Rhodospirillum rubrum* was used to produce hydrogen. *R. rubrum* has demonstrated ability to ferment various substrates e.g., lactate, whey or yogurt to hydrogen gas (Zurrer, Bachofen, 1982; Venkataraman, Vatsala, 1990). It was also reported to be capable of improving COD of distillery wastewater (Vatsala, Ramasamy, 1987). To our knowledge, there is no information on using mixed cultures of anaerobic mixed culture from anaerobic treatment pond and/or photosynthetic bacteria to produce hydrogen from cassava starch manufacturing wastewater. The advantages of using mixed cultures over pure culture including (i) lower cost as no sterile condition is needed, (ii) non-sterile organic wastes can be used as substrate and (iii) possibility for operation control based on differential kinetics of microbial subgroups (Levin et al., 2004).

Therefore, this study aimed to investigate a production of biohydrogen from cassava starch-manufacturing wastewater by anaerobic fermentation process. The experiment was divided into two parts. Part one was conducted to investigate biohydrogen production from cassava starch manufacturing wastewater in a series batch test using (i) anaerobic sludge, (ii) co-culture of anaerobic sludge and *R. rubrum* and (iii) a sequential addition of anaerobic sludge and *R. rubrum*. In part two, a continuously fermentation process i.e., UASB system was develop for sustainable hydrogen production from cassava wastewater by developed granulated sludge. Information from this study will be useful when applied to the recovery of bioenergy, hydrogen gas, from wastes derived from renewable resources.

## **2. Biohydrogen Production from Cassava Starch Manufacturing Wastewater in a Series Batch Test**

A series of batch tests were conducted to investigate the biohydrogen production potential from cassava starch manufacturing wastewater at 20,000 mg-COD/L using three different inocula, namely (i) anaerobic sludge, (ii) co-culture of anaerobic sludge and *Rhodospirillum rubrum* and (iii) a sequential addition of anaerobic sludge and *R. rubrum*. Cassava starch manufacturing wastewater was obtained from Asia Modify Starch Factory, Kalasin Province, Thailand. The pH value of the wastewater was  $5.02 \pm 0.35$  and the total COD was  $22,600 \pm 2,750$  mg COD/L. The wastewater used in all experiments had COD value of 20,000 mg-COD/L and was adjusted to the final COD:N:P ratio of 100:10:1. The N and P source to obtain the required ratio were 6.7 g/L  $\text{NH}_4\text{Cl}$  and 0.8 g/L  $\text{K}_2\text{HPO}_4$ , respectively. Anaerobic sludge obtained from the municipal anaerobic digester in Ube City, Yamaguchi, Japan had volatile suspended solid (VSS) and total suspended solid (TSS) of 13,300 and 19,300 mg/L, respectively. This sludge showed amylase activity by hydrolyzing starch using the method described previously (Yokoi et al., 1998). *R. rubrum* ATCC 11170 was purchased from the DSMZ–Deutsche Sammlung van Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

Biohydrogen production by anaerobic sludge was firstly conducted to investigate the ability of microorganisms in anaerobic sludge to produce hydrogen from cassava starch manufacturing wastewater at various initial pH and cultivation temperatures. A series of batch tests was conducted in a 75 -mL glass serum bottle with a working volume of 50 mL.

Ten mL of the pretreated anaerobic sludge was inoculated to 40 mL of the wastewater in a serum bottle. The initial pH was adjusted to 5.0, 6.0 or 7.0 using 1N HCl or 1N NaOH. The gas phase was then purged with argon to create anaerobic condition. Each experiment set conducted in a temperature-controlled water bath at two mesophilic temperatures of 30 and 35°C and thermophilic temperatures of 45 and 55 °C.

The volume of biogas was measured by plunger displacement method using wetted glass syringes. The components of biogas were analyzed using a gas chromatograph (GC) (Model 8APT, SHIMADZU, Japan) equipped with a thermal conductivity detector (TCD). A 3m x 3mm stainless-steel column packed with 60/80 mesh activated charcoal (Model 8APF, SHIMADZU, Japan) was used to analyze the percentage of hydrogen, nitrogen, methane and carbon dioxide in the biogas produced. Argon was used as the carrier gas at a flow rate of 70 mL/min. The temperatures of injector, detector and column were 50, 50 and 60°C, respectively. The concentrations of acetic, propionic and n-butyric acids were determined by a gas chromatography (Model 8A, SHIMADZU, Japan) equipped with a flame ionization detector (FID) and a 3 m x 3.2 mm glass column packed with 30/60 mesh Unisole F-200 (GL Science Inc. Japan). Injector, detector and column temperatures were at 250, 140 and 140°C, respectively. Nitrogen, hydrogen and pressured air were used as carrier gases with flow rate of 50, 60 and 500 mL/min, respectively. The cumulative hydrogen production in the anaerobic batch experiments using anaerobic sludge followed the modified Gompertz equation (Fang, Zhang, 2005): Results indicated that using anaerobic sludge alone, the maximum specific hydrogen production of 1,511 mL H<sub>2</sub>/g-VSS and the maximum hydrogen yield of 251 mL/g-COD fed were obtained at thermophilic temperature (55°C) and initial pH 5.0.

Biohydrogen production by co-culture of anaerobic sludge and *R. rubrum* was then investigated in batch fermentation. Ten mL of anaerobic sludge and 10 mL of 0.68 gram dry cell/L *R. rubrum* suspension were inoculated in 30 mL of the wastewater prepared as previously described in 75-mL serum bottle. The initial pH of liquid contents was adjusted to pH 5.0, 6.0 and 7.0 with 1 N HCl or 1 N NaOH. After the bottles were purged with argon, they were incubated at 30°C without a pH control under illumination at 6,000 lux by the fluorescent lamp. The produced biogas and VFAs concentration in fermentative broth were analyzed by using methods described above. Results showed that the use of co-culture of

anaerobic sludge and *R. rubrum* in single stage as inoculum improved the specific hydrogen production by 1.7-fold and the hydrogen yield by 1.7-fold in comparison to the use of anaerobic sludge alone at the same test conditions of 30°C and initial pH 7.0.

Hydrogen production from wastewater by sequential addition of anaerobic sludge and *R. rubrum* was operated in order to enhance hydrogen production efficiency in batch fermentation. After inoculating 10 ml of anaerobic sludge in 40 ml of the wastewater, prepared as previously described, with various initial pH of 5.0, 6.0 and 7.0 and culturing for 110 h, the anaerobic sludge of each initial pH was filtered out through the 47-mm glass fiber filter, (Toyo Roshi Kaisha, Ltd.). Ten mL of 0.68 g dry cell/L *R. rubrum* suspension was then added to the remaining 40 mL of the culture broth in the serum bottle and the pH was adjusted to pH 7 using 1N NaOH or 1 N HCl. The head space was then replaced with argon and the serum bottles were further incubated at 30°C without a pH control under illumination at 6000 lux by the fluorescent lamp. Superior results were obtained when a sequential addition of anaerobic sludge and *R. rubrum* was used for hydrogen production. The cumulative hydrogen of 300 mL with COD-H<sub>2</sub>/COD<sub>input</sub> of 0.28 was produced at 30°C and initial pH 7.0. This study indicated that cassava starch manufacturing wastewater has a potential for sustainable hydrogen production.

### **3. Bio-Hydrogen Production from Cassava Wastewater by Upflow Anaerobic Sludge Blanket Reactor**

Sustainable hydrogen production from cassava wastewater was conducted in Upflow Anaerobic Sludge Blanket (UASB) reactor. Cassava starch manufacturing wastewater was obtained from Asia Modify Starch Factory, Kalasin Province, Thailand. The pH value of the wastewater was 5.02±0.35 and the total COD was 22,600±2,750 mg COD/L. After removal of suspended solids by simple gravity settling, cassava wastewater was used as a feed at the concentration of 10,000-14,000 mg COD/L. The wastewater was added with 6.7 g/L of NH<sub>4</sub>Cl as nitrogen source and 0.8 g/L of K<sub>2</sub>HPO<sub>4</sub> as phosphorous source. In addition, KCl 0.0075 g/L, MgCl<sub>2</sub>·6H<sub>2</sub>O 0.0081 g/L, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.0025 g/L, FeCl<sub>3</sub>·6H<sub>2</sub>O 0.0042 g/L, CoCl<sub>2</sub>·6H<sub>2</sub>O 0.00018 g/L and CaCl<sub>2</sub>·6H<sub>2</sub>O 0.015 g/L were added as trace elements for the anaerobic microorganisms.

Inoculum used to form UASB granule was anaerobic sludge obtained from the alcoholic factory, Khon Kaen, Thailand. It was collected from a final sedimentation tank and then filtered through a 1,000  $\mu\text{m}$  mesh screened to separate out the large particulate matters. The sludge had the pH, total solids (TS) and volatile suspended solids (VSS) of 6.4, 23,500 mg/L and 19,400 mg/L, respectively. Sludge was heat-treated at 100°C for 30 min in order to inhibit the methanogenic bacteria prior the usage as inocula for hydrogen production in the UASB process.

The 9.4-L UASB reactor was operated at the ambient temperature and pH was controlled to be in the range of 6.0-6.5 by adding  $\text{NaHCO}_3$  at a concentration of 2.4 g/L as the buffer. Working volume, interior diameter and height of this UASB reactor were 9.4 L, 9.5 cm, and 630 cm, respectively. The sampling ports were designed at the interval of 10 cm along the reactor tank. An operation of UASB was started by filing the reactor with 5 L of 19,400 mg VSS/L of the heat-treated (resulted in approximately 10,000 mg VSS/L sludge). Cassava wastewater was fed up gradient continuously through inoculated heat-treatment sludge in the reactor with the HRT of 24 h (0.4 L/h) for 62 days to allow granules formation. After the granules were developed, the reactor was operated at HRT 24 to reach a steady state condition judged from stable values of COD present in the UASB reactor. After reached the steady state at HRT 24, the HRT reduction was conducted in a stepwise reduction through 18 (an influent velocity of 0.5 L/h), 12 (0.8 L/h), 8.4 (1.0 L/h) and 4.8 (2.0 L/h) hrs, respectively. The effluent and produced gas from the UASB reactor were sampled once a day to determine COD, volatile fatty acid (VFA), total suspended solid (TSS) and volatile suspended solid (VSS) concentration, gas production and gas compositions. Biomass was periodically taken from the sampling ports at the bed zone and blanket zone of the reactor to determine the VSS concentration according to the Standard Methods of APHA (1995). The volume of biogas production was measured by gas meter and the analysis of biogas composition was performed using a gas chromatograph (GC) (Model 2014, SHIMADZU, Japan) equipped with a thermal conductivity detector (TCD). A 2 m x 2.5 mm stainless-steel column packed with 60/80 mesh Unibead C was used to analyze the content of hydrogen, nitrogen, methane and carbon dioxide in the biogas produced. Helium was used as the carrier gas at a flow rate of 25 ml/min. The temperature of injector, detector and column were 150, 150 and 145°C, respectively. Concentrations of acetic, propionic and butyric acids were

determined by a gas chromatography (Model 14B, SHIMADZU, Japan) equipped with a flame ionization detector (FID) and a 3 m x 3.2 mm glass column packed with 30/60 mesh Cabowax 20M. Injector, detector and column temperatures were at 200, 250 and 180°C, respectively. Nitrogen, hydrogen and pressured air were used as carrier gases with flow rate of 80, 70 and 500 mL/min, respectively.

Results revealed that the biomass concentration in the bed zone of the UASB reactor was increased throughout the experiment suggesting that hydrogen producing granulated sludge were developed. A shift in HRT from 24 h to 12 h appeared to enhance hydrogen production rate. When HRT was shortened from 12 h to 4.8 h the percentage of hydrogen produced decreased from 46% to 10%. Peak of the hydrogen yield of 46 mL H<sub>2</sub>/g COD and the hydrogen production rate of 16.1 L/d were obtained at HRT 12 h. These results indicated that HRT 12 h was an optimum HRT in producing hydrogen from cassava wastewater. An average granular sludge at HRT 12 h was a light-grey in color and was 0.14 mm in diameter. Each gram of biomass produced 0.89 mL H<sub>2</sub>/day with gas evolved mixture of 46% hydrogen, 40% carbon dioxide and less than 2% methane. The effluent volatile suspended solid, the endogenous decay coefficient (K<sub>d</sub>) and yield coefficient (Y<sub>g</sub>) of hydrogen producer granules were 235 mg/L and 0.64 /day and 0.93 g VSS/g COD, respectively. During the efficient hydrogen production stage, a major soluble metabolite was butyric acid, followed by acetic acid and propionic acid.

The granulated sludges were sampled at each HRT (24, 18, 12, 8.4 and 4.8 h) for microbial analysis. Granules were firstly centrifuged at 14,000g for 30 sec to remove the fermentative broth. The granular pellets were then washed with a pH 7.4 phosphate buffer before centrifugation at 14,000g for 30 sec to remove washing buffer. The washed granules were then sonicated for 10 sec in ice box. The total community DNA from the granulated sludge was extracted on beading method with Fast DNA Spin kit of soil, Bio 101 (Biospec Products, Bartlesville, USA), followed by PCR amplification using the primer set of 357F-GC (5'-CGCCCGCCGCGCGCGGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3') and 518 R (5'-GTATTACCGCGGCTGCTGG-3') with an annealing temperature of 72 °C in automated thermal cycle (Biorad, USA). The PCR amplified products were then screened using DGGE to investigate the microbial population variations under different HRT. A 40% acrylamide/Bis solution was used to cast a gel with denaturant

gradients ranging 30-70%. Electrophoresis was conducted in a 1xTAE buffer solution at 130 V and 60°C for 5 hr. The 16S rDNA bands on the gel were then stained with ethidium bromide. The repeat PCR of DNA in presenting band was conducted 5 times in order to purify the DNA. The obtained PCR products were sequenced by Biomolecular Analysis Service Unit, Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Thailand. The nearly full length sequences were compared with the reference microorganisms available in the GenBank by BLAST search ([www.NCBI.com](http://www.NCBI.com)). The DGGE profiles clearly showed that the microbial community changed with HRT. The more number of bands was observed in the granule obtained from the UASB process operated at higher HRT. All of appeared bands were identified for microbial species. Dominant species found in bands at every HRT were *Megasphaera elsdenii* and *Megasphaera hominis* which had similarity percentages of 100% and 99%, respectively. These two species were reported as the hydrogen producing bacteria in the genus of Clostridia which were well known as hydrogen producer.

#### 4. Conclusions

Conclusions drawn from this study are as follows:

- 1) Maximum hydrogen production in batch culture of anaerobic sludge was achieved at 55 °C and initial pH 5.0 with a specific hydrogen production of 1,511 mL H<sub>2</sub>/g-VSS and a hydrogen yield of 251 mL/g-COD.
- 2) Results from hydrogen production by co-culture showed that the presence of *R. rubrum* improved the specific hydrogen production by 1.7 folds and the hydrogen yield by 1.7 folds when compared to the use of anaerobic sludge alone at the same conditions of 30°C and initial pH 7.0.
- 3) Superior results were obtained when a sequential addition of anaerobic sludge and *R. rubrum* was used to ferment hydrogen from cassava starch manufacturing wastewater. Hydrogen yield in terms of COD-H<sub>2</sub>/COD<sub>input</sub> of 0.28 and the cumulative hydrogen of 300 mL were obtained at 30°C and initial pH 7.0.
- 4) UASB process was effectively applied in hydrogen production from cassava wastewater by heat treated anaerobic sludge as inoculum.

- 5) The biomass concentration in the bed zone of the UASB reactor increased throughout the experiment suggested that the hydrogen producer granular were developed.
- 6) HRT 12 h was the most optimum HRT for hydrogen production from cassava wastewater indicated by the highest hydrogen yield and total hydrogen production of 46 mL H<sub>2</sub>/g COD and 16.1 L/day, respectively, and relatively low the washed out sludge discharge rate of 7,896 mg/day. Each gram of biomass produced 0.89 mL H<sub>2</sub>/day with gas evolved mixture of 46% hydrogen, 40% carbon dioxide and less than 2% methane.
- 7) Analysis of microbial by DGGE in granulated sludge indicated that dominant species found in each band on the DGGE profile at every HRT were *Megasphaera elsdenii* and *Megasphaera honisis*.

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## RESEARCH CONTENTS

(Prepared as Manuscripts)

### PART I

#### **Biohydrogen Production from Cassava Starch Manufacturing Wastewater**

A paper submitted to publish in the Biomass Bioenergy (Manuscript code 06-174)

##### **Abstract**

A series of batch tests were conducted to investigate the biohydrogen production potential from cassava starch manufacturing wastewater at 20,000 mg-COD/L using three different inocula, namely (i) anaerobic sludge, (ii) co-culture of anaerobic sludge and *Rhodospirillum rubrum* and (iii) a sequential addition of anaerobic sludge and *R. rubrum*. Using anaerobic sludge alone, the maximum specific hydrogen production of 1,511 mL H<sub>2</sub>/g-VSS and the maximum hydrogen yield of 251 mL/g-COD fed were obtained at thermophilic temperature (55°C) and initial pH 5.0. The use of co-culture of anaerobic sludge and *R. rubrum* in single stage as inoculum improved the specific hydrogen production by 1.7-fold and the hydrogen yield by 1.7-fold in comparison to the use of anaerobic sludge alone at the same test conditions of 30°C and initial pH 7.0. Superior results were obtained when a sequential addition of anaerobic sludge and *R. rubrum* was used for hydrogen production. The cumulative hydrogen of 300 mL with COD-H<sub>2</sub>/COD<sub>input</sub> of 0.28 was produced at 30°C and initial pH 7.0. This study indicated that cassava starch manufacturing wastewater has a potential for sustainable hydrogen production.

*Keywords:* anaerobic sludge; biohydrogen production; cassava starch manufacturing wastewater; *Rhodospirillum rubrum*

## 1. Introduction

Thailand is the world's largest exporter of cassava, contributes to about 80 percent of total world exports [1]. Cassava is a major source of starch and about 18 million tons of starch are processed annually from cassava [2]. Different constituents present in the cassava are shown in Table 1 [3].

**Table 1** Constituents present in the cassava [3]

Constituents	%
Moisture	69.8
Starch	22.0
Sugars	5.1
Protein	1.1
Fats	0.4
Fiber	1.1
Ash	0.5

Wet processing is commonly adopted for the recovery of starch from cassava roots. However, the process generates large volume of high strength liquid stream, which is regarded as wastewater. One kilogram of fresh cassava roots yields about 0.2 kg of starch, 0.4-0.9 kg of cake and about 5-7 L of wastewater [4]. The wastewater has a very high chemical oxygen demand (COD), biochemical oxygen demand (BOD) and total solid as shown in Table 2 [5].

**Table 2** Important characteristics of wastewater from cassava starch factories [5]

Component	Content
CODtotal	13,000-19,500 mg/L
CODsoluble	6,500-13,600 mg/L
BODtotal	6,500-12,600 mg/L
Total solid	12,500-20,000
Total soluble solid	5,800-8,000

Disposal of high strength cassava starch manufacturing wastewater poses a burden on the environment. Aerobic wastewater treatment which is commonly adopted to treat the wastewater requires large energy input to provide aeration due to high COD. Anaerobic treatment of cassava starch manufacturing wastewater which yields methane has been well investigated by various researches [6-12]. However, methane is a low value end-product with relatively less energy content (about 56 kJ/g CH<sub>4</sub>). Furthermore, methane and its combustion by-product are powerful greenhouse gases and responsible for global climate change.

Biological hydrogen production through fermentation represents a new area of bioenergy production that could substitute non-renewable fossil fuels. To our best understanding, biohydrogen production from cassava starch manufacturing wastewater has never been investigated. Biohydrogen production has many inherent merits including availability of naturally occurring microbes, a shorter hydraulic retention time (HRT), a simple bioreactor system, and potential for waste remediation. Biohydrogen production can be classified into 5 processes which are direct biophotolysis, indirect biophotolysis, biological water-gas shift reaction, dark fermentation and photo-fermentation [13]. Among these 5 processes, fermentation receives much attention in hydrogen production in recent years due to its feasibility [14]. In general, the amount of hydrogen produced by dark fermentation is lower than that of photo-fermentation and the production rate is slower [15,16]. However, dark fermentation has advantages over photo-fermentation including independence of solar radiation, less space requirement and independence of weather condition [14].

Sequential dark and photo-fermentation and combined dark and photo-fermentations for hydrogen production are new approaches in bio-hydrogen production. In such system, anaerobic fermentation of organic wastes produces organic acids such as acetic and butyric acids which are the intermediates for hydrogen production by photosynthetic bacteria [17]. Advantages of sequential dark and photo-fermentation system over single stage dark or photo-fermentation processes include (i) sufficient availability of organic acids from dark fermentation and (ii) better effluent quality due to the use of organic acids by photo-fermentative bacteria [18].

Sequential dark-photo fermentation technique was successfully used to produce hydrogen from residual carbohydrates such as sweet potato starch residue by *Clostridium*

*butyricum*, *Enterobacter aerogenes* and *Rhodobacter sp.* M-19 [19]; potato starch residue by *C. butyricum*, *E. aerogenes* and *Rhodobacter sp.* M-19 [20]; algal biomass (*D. tertiolecta*) by *Lactobacillus amylovorus* and *R. marinum* A-501 [21] and solid waste by mixed anaerobic culture and *R. sphaeroides* RV [22].

Combined dark and photo-fermentation was reported to produce higher yield of hydrogen than the single stage. A report has shown that co-culture of *C. butyricum* and *Rhodobacter sp.* in starch produced higher hydrogen yield (4.5 mol/mol glucose) than single stage dark fermentation (1.9 mol/mol glucose) and sequential two steps fermentation (3.7 mol/mol glucose) [23]. Co-cultures of *R. marinum* and *V. fluvialis* were reported to produce higher hydrogen yields than the use of *R. marinum* alone [24].

In the present study, a photosynthetic bacteria *Rhodospirillum rubrum* was used to produce hydrogen. *R. rubrum* has demonstrated an ability to ferment various substrates e.g., lactate, whey or yogurt to hydrogen gas [25-27]. It was also reported to be capable of improving COD of distillery wastewater [28]. To our knowledge, there is no information on using mixed cultures of anaerobic mixed culture from anaerobic treatment pond and/or photosynthetic bacteria to produce hydrogen from cassava starch manufacturing wastewater. The advantages of using mixed cultures over pure culture including (i) lower cost as no sterile condition is needed, (ii) non-sterile organic wastes can be used as substrate and (iii) possibility for operation control based on differential kinetics of microbial subgroups [13]. Therefore, this study aimed to investigate a production of biohydrogen from cassava starch-manufacturing wastewater using (i) anaerobic sludge, (ii) co-culture of anaerobic sludge and *R. rubrum* and (iii) a sequential addition of anaerobic sludge and *R. rubrum*. Information from this study will be useful when applied to the recovery of bioenergy, hydrogen gas, from wastes derived from renewable resources.

## 2. Materials and Methods

### 2.1 Wastewater

Cassava starch manufacturing wastewater was obtained from Asia Modify Starch Factory, Kalasin Province, Thailand. The pH value of the wastewater was  $5.02 \pm 0.35$  and the total COD was  $22,600 \pm 2,750$  mg COD/L. Other compositions were shown in Table 3. The wastewater used in all experiments had COD value of 20,000 mg-COD/L and was adjusted

### **2.3 Hydrogen production by anaerobic sludge**

This experiment was conducted to investigate the ability of microorganisms in anaerobic sludge to produce hydrogen from cassava starch manufacturing wastewater at various initial pH and cultivation temperatures. A series of batch tests was conducted in a 75 -mL glass serum bottle with a working volume of 50 mL. Ten mL of the pretreated anaerobic sludge was inoculated to 40 mL of the wastewater in a serum bottle. The initial pH was adjusted to 5.0, 6.0 or 7.0 using 1N HCl or 1N NaOH. The gas phase was then purged with argon to create anaerobic condition. Each experiment set conducted in a temperature-controlled water bath at two mesophilic temperature of 30 and 35°C and thermophilic temperatures of 45 and 55 °C.

### **2.4 Hydrogen production by co-culture of anaerobic sludge and *R. rubrum***

This experiment was designed to examine the use of co-culture of anaerobic sludge and *R. rubrum* for hydrogen production. Ten mL of anaerobic sludge and 10 mL of 0.68 gram dry cell/L *R. rubrum* suspension were inoculated in 30 mL of the wastewater prepared as previously described in 75-mL serum bottle. The initial pH of liquid contents was adjusted to pH 5.0, 6.0 and 7.0 with 1 N HCl or 1 N NaOH. After the bottles were purged with argon, they were incubated at 30°C without a pH control under illumination at 6,000 lux by the fluorescent lamp.

### **2.5 Hydrogen production by a sequential addition of anaerobic sludge and *R. rubrum***

In this system we intended to enhance a production of hydrogen from wastewater by sequential addition of anaerobic sludge and *R. rubrum*. After inoculating 10 ml of anaerobic sludge in 40 ml of the wastewater, prepared as previously described, with various initial pH of 5.0, 6.0 and 7.0 and culturing for 110 h, the anaerobic sludge of each initial pH was filtered out through the 47-mm glass fiber filter, (Toyo Roshi Kaisha, Ltd.). Ten mL of 0.68 g dry cell/L *R. rubrum* suspension was then added to the remaining 40 mL of the culture broth in the serum bottle and the pH was adjusted to pH 7 using 1N NaOH or 1 N HCl. The head space was then replaced with argon and the serum bottles were further incubated at 30°C without a pH control under illumination at 6000 lux by the fluorescent lamp.

to the final COD:N:P ratio of 100:10:1. The N and P source to obtain the required ratio were 6.7 g/L  $\text{NH}_4\text{Cl}$  and 0.8 g/L  $\text{K}_2\text{HPO}_4$ , respectively.

**Table 3** Composition of cassava wastewater from Asia Modify starch Factory. Data are given as mean $\pm$ SD, n = 3

Parameter	mg/L
COD	22,600 $\pm$ 2,750
Nitrogen	258 $\pm$ 92
Phosphorus	54 $\pm$ 24
Total suspended solid	1,193 $\pm$ 230
Volatile suspended solid	1,300 $\pm$ 316
Acetic acid	191 $\pm$ 48
Propionic acid	16 $\pm$ 3
Butyric acid	63 $\pm$ 17
pH	5.02 $\pm$ 0.35

## 2.2 Seed Inocula

Anaerobic sludge was obtained from the municipal anaerobic digester in Ube City, Yamaguchi, Japan. The sludge was filtered through a 1,000  $\mu\text{m}$  mesh screen and stored at 4°C prior to usage. Ten mL of the sludge was washed by centrifuging at 2,400G for 10 min at 4°C and then re-suspended in 10 mL of sterile milli-Q water before inoculation.

The sludge had volatile suspended solid (VSS) and total suspended solid (TSS) of 13,300 and 19,300 mg/L, respectively. The sludge showed amylase activity by hydrolyzing starch using the method described previously [29].

*R. rubrum* ATCC 11170 was purchased from the DSMZ–Deutsche Sammlung van Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. The cell suspension of *R. rubrum* 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%  $\text{K}_2\text{HPO}_4$ , 0.5%  $\text{MgSO}_4$ , 10% yeast extract, 2.01% DL-malic acid and 0.33% DL-glutamic acid.

## 2.6 Analytical method

### 2.6.1 Gas Analysis

The volume of biogas was measured by plunger displacement method using wetted glass syringes [30]. The components of biogas were analyzed using a gas chromatograph (GC) (Model 8APT, SHIMADZU, Japan) equipped with a thermal conductivity detector (TCD). A 3m x 3mm stainless-steel column packed with 60/80 mesh activated charcoal (Model 8APF, SHIMADZU, Japan) was used to analyze the percentage of hydrogen, nitrogen, methane and carbon dioxide in the biogas produced. Argon was used as the carrier gas at a flow rate of 70 mL/min. The temperatures of injector, detector and column were 50, 50 and 60°C, respectively.

### 2.6.2 Volatile Fatty Acids (VFAs) Analysis

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

### 2.6.3 Kinetic analysis

Volume of biogas produced was calculated by using the following mass balance equation [31]:

$$V_{H,i} = V_{H,i-1} + C_{H,i} (V_{G,i} - V_{G,i-1}) + V_H (C_{H,i} - C_{H,i-1}) \quad (1)$$

where  $V_{H,i}$  and  $V_{H,i-1}$  are the cumulative hydrogen gas volumes at the current and previous time, respectively;  $V_{G,i}$  and  $V_{G,i-1}$  are total biogas volume at the current and previous time, respectively;  $C_{H,i}$  and  $C_{H,i-1}$  are the fraction of hydrogen gas in the headspace at the current and previous time, respectively;  $V_H$  is the volume of headspace.

The cumulative hydrogen production in the anaerobic batch experiments using anaerobic sludge followed the modified Gompertz equation [32]:

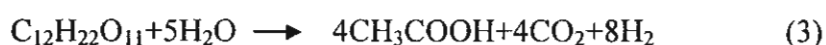
$$H(t) = P \exp \left\{ - \exp \left[ \frac{R_m e (\lambda - t) + 1}{P} \right] \right\} \quad (2)$$

$H(t)$  is the cumulative volume of hydrogen produced (mL),  $P$  is the hydrogen production potential (mL),  $R_m$  is the maximum production rate (mL/h),  $e$  is 2.71828 and  $\lambda$  is the lag time (h) and  $t$  is time (h). The maximum specific hydrogen production rate (mL/g-VSS.h) was calculated by dividing  $R_m$  by the initial sludge VSS. The hydrogen yield or conversion efficient (mL/g-COD wastewater) was calculated by dividing  $P$  by the g-COD waste water. The specific hydrogen production (mL H<sub>2</sub> /g-VSS) was calculated by dividing  $P$  by g-VSS.

### 3. Results and discussion

#### 3.1 Hydrogen production from cassava starch manufacturing wastewater by anaerobic sludge

Results from cultivating the sludge at mesophilic (30 and 35°C) and thermophilic (45 and 55°C) temperatures showed that the main organic acid produced at mesophilic temperatures was acetic acid (HAc) (Table 4) suggesting that the reaction was a HAc fermentation type. Normally fermentation products of acidogenic fermentation is butyric acid (HBu) [33]. However, different fermentation products could be obtained if the culture conditions of the bacteria groups contributing to the fermentation process are changed [34-37]. Results also indicated that more HAc was produced at initial pH 5 > 6 > 7 at mesophilic temperature (Table 4). This can be explained using Equation (3) by which higher HAc was produced as the reaction was driven towards acetate production at lower pH.



At thermophilic temperatures, HAc and HBu were major fermentation products except at initial pH 5 and 55°C that 94.3 % of HBu was obtained. *Clostridium* species might be responsible for high percentage of HBu production at these conditions. The species are known for butyrate fermentation [38, 39]. There was a report indicated that *Clostridium* species fermented sugars, starch and butyrate, from which acetate, CO<sub>2</sub> and O<sub>2</sub> were the products obtained [40]. *C. aceticum* can reduce CO<sub>2</sub> to acetate with H<sub>2</sub> 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 shown in Table 4 that a very small amount of HPr was generated in our experiments. HPr generated during the experiment might be resulted from symbiotic nature or syntrophic interactions of the microorganisms in mixed culture [41]. To support our speculation that *Clostridium* species is the dominant microorganism at thermophilic temperatures, we tested the amylase activity of the *Clostridium* species in anaerobic sludge by hydrolyzing the starch and found that the anaerobic sludge had amylase activity of  $8.5 \times 10^7$  CFU/mL. Valdez-Vazquez *et al.* [42] stated that in the anaerobic mixed cultures, bacteria of *Clostridia* genera are present in great proportion. Due to above reasons we concluded that the dominant microorganism in our anaerobic sludge at thermophilic temperature was from *Clostridia* genera. More investigation on identifying the microorganisms using microbiological method such as API system (bioMérieux sa, France) or molecular biology techniques e.g. Fluorescence In Situ Hybridization (FISH) or Denaturing Gradient Gel Electrophoresis (DGGE) to study microbial community in anaerobic sludge should be conducted.

Final pH dropped below pH 5 for each experimental condition (Table 4). This may result from the VFAs produced in each serum bottles. The effectiveness of H<sub>2</sub> production was normally indicated by H<sub>Bu</sub>/H<sub>Ac</sub> ratio [38]. Results revealed that for the higher H<sub>Bu</sub>/H<sub>Ac</sub> ratio (Table 4), a higher maximum specific hydrogen production rate was obtained (Table 5). Different optimal H<sub>Bu</sub>/H<sub>Ac</sub> ratios for H<sub>2</sub> production could result from the differences in the anaerobic cultures and the substrate used [39]. For instance, the optimal H<sub>Bu</sub>/H<sub>Ac</sub> ratios for *C. butyricum* and *Butyribacterium methylotrophicum* fermentation were reported to be 2 and 0.75, respectively. In our experiment, we found that the maximum H<sub>Bu</sub>/H<sub>Ac</sub> for production of H<sub>2</sub> from cassava starch manufacturing wastewater by anaerobic sludge was 18.01 at initial pH 5 and 55°C (Table 4).

This result corresponded to the maximum hydrogen production potential (P). The highest hydrogen yield and the highest specific hydrogen production obtained were 201 mL, 251 mL/g-COD and 1,511 mLH<sub>2</sub> /g-VSS, respectively (Table 5). These results indicated that an optimum condition for hydrogen production from cassava starch manufacturing wastewater by anaerobic sludge was initial pH 5 and 55°C.

**Table 4** Effect of initial pH of cassava wastewater and cultivation temperature on production of VFAs. Data are given as mean $\pm$ SD, n = 2

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

Data also indicated that thermophilic temperature was more suitable to produce hydrogen from cassava starch manufacturing wastewater by anaerobic sludge than mesophilic temperature (Table 5). We observed that, at thermophilic temperature, when the P values were high the  $R_m$  values were also high. This may be due to the fact that microorganisms in the sludge possess a range of responsive capacities for different adverse circumstances resulting in a certain characteristic, such as P,  $R_m$  and  $\lambda$  [41]. The  $\lambda$  values at thermophilic range were higher than at mesophilic range except at initial pH 7 and 55°C indicating that sludge microorganisms needed a longer lag time to adjust to a new environment at high temperature.

**Table 5** Modified Gompertz equation parameters for hydrogen production from cassava wastewater, 20,000 mg/L, by anaerobic sludge.

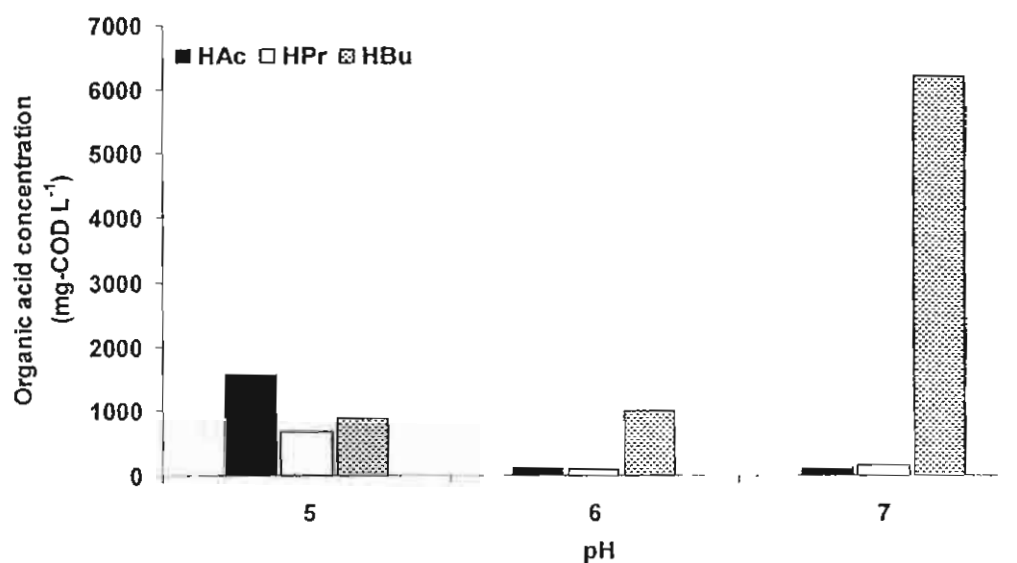
Initial pH	T (°C)	$\lambda$ (h)	$R_m$ (mL/h)	$P$ (mL)	Maximum specific H <sub>2</sub> production rate (mL/g-VSS-h)	Specific hydrogen production (mL H <sub>2</sub> /g-VSS)	Hydrogen yield (mL/g COD)	R <sup>2</sup>
5	30	3	0.1	4	0.98	30	5	0.95
5	35	8	0.06	8	0.45	60	10	0.90
5	45	130	4	130	30.06	977	163	0.97
5	55	201	1	201	8.27	1,511	251	0.99
6	30	8	0.2	8	1.50	60	10	0.96
6	35	29	0.06	29	0.45	218	36	0.92
6	45	127	5	127	37.59	955	159	0.97
6	55	87	1	87	7.51	654	109	0.97
7	30	58	2	58	15.04	436	73	0.99
7	35	191	1	191	4.89	1,436	239	0.95
7	45	156	7	156	52.63	1,173	195	0.96
7	55	32	0.4	32	3.00	241	40	0.90

### 3.2 Hydrogen production from cassava starch manufacturing wastewater by co-culture of anaerobic sludge and *R. rubrum*

This experiment examined the ability of anaerobic sludge and *R. rubrum* to co-produce hydrogen from cassava starch manufacturing wastewater in a single stage system at various initial pH and a mesophilic temperature of 30°C which is an optimal temperature for *R. rubrum*. Fig. 1 depicts that HBU was the major intermediate organic acid at each initial pH suggesting a butyrate fermentation pathway. The highest percentage of HBU (Fig. 1) and the maximum P value were obtained at the initial pH 7 (Table 6) indicating that volume of hydrogen gas produced depended on the concentration of butyric acid.

**Table 6** Modified Gompertz equation parameters for hydrogen production from cassava wastewater, 20,000 mg/L, by co-culture of anaerobic sludge and *R. rubrum*.

Initial pH	T (°C)	$\lambda$ (hr)	$R_m$ (mL/h)	$P$ (mL)	Maximum specific H <sub>2</sub> production rate (mL/g-VSS-h)	Specific hydrogen production (mL H <sub>2</sub> /g-VSS)	Hydrogen yield (mL/g COD)	R <sup>2</sup>
	30	13	1.6	80	11.81	598	100	0.95
	30	16	1.8	86	13.23	647	108	0.97
	30	13	2	100	15.26	749	125	0.97



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

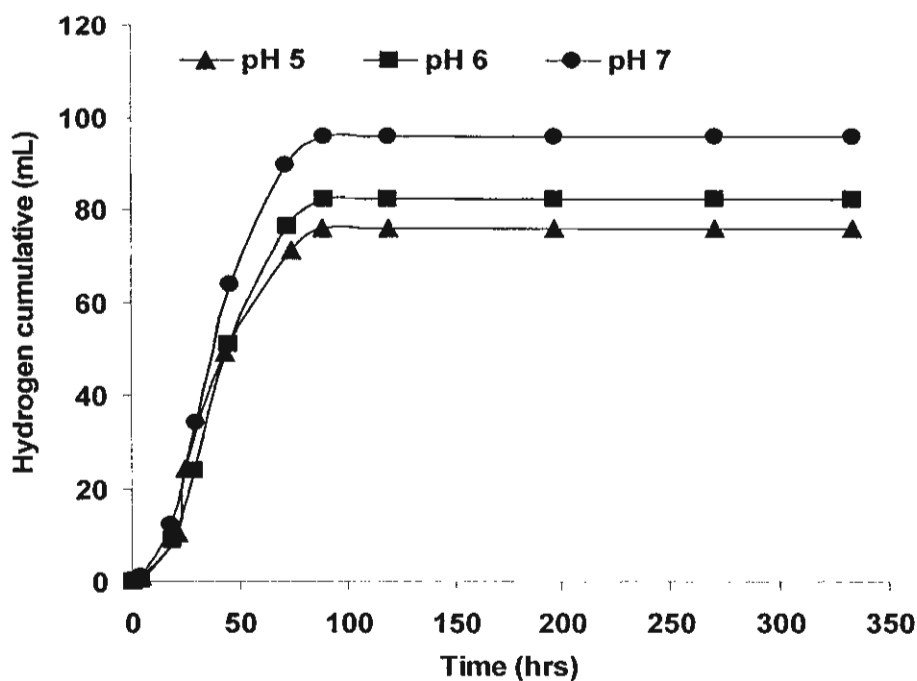


Fig. 2. Hydrogen cumulative by co-culture of anaerobic sludge and *R. rubrum*.

The presence of *R. rubrum* improved the specific hydrogen production by 1.7-fold and the hydrogen yield by 1.7-fold (Table 6) when compared to the use of anaerobic sludge at the same conditions of 30°C and initial pH 7.0 (Table 5), indicating that *R. rubrum* was effective in producing hydrogen. It is worth to note that hydrogen gas volume produced by co-culture at initial pH 5, 6 and 7 at 30 °C were higher (Table 6 and Fig. 2) than that obtained by anaerobic sludge alone (Table 5). Low hydrogen gas volume might be the result of hydrogen consumption by hydrogen consuming bacteria present in the sludge. In anaerobic digestion plant, a consortium of microorganisms converts organic waste into a mixture of CH<sub>4</sub> and CO<sub>2</sub>. Prior to the methanogenic stage, hydrogen was produced as an intermediate product and was utilized as it was produced by methanogenic archaea, acetogenic bacteria and sulfate reducing bacteria [42]. Therefore, if the hydrogen consuming bacteria can be inhibited, the more hydrogen would be accumulated. In addition, *R. rubrum* might not be able to out-compete 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[42].

### 3.3 Hydrogen production from cassava starch manufacturing wastewater by a sequential addition of anaerobic sludge and *R. rubrum*

Sequential dark and photo-fermentation is a new approach in biological hydrogen gas production. In this system we intended to enhance a production of hydrogen from cassava starch manufacturing wastewater by allowing the anaerobic sludge to convert starch in the wastewater to organic acids and then using *R. rubrum* to produce hydrogen from organic acids obtained from the previous step. We adjusted the pH of the culture broth from below pH 5.0 to pH 7.0 before adding *R. rubrum* into the culture broth. This was because no H<sub>2</sub> gas would be produced without pH adjustment (data not shown) suggesting that *R. rubrum* cannot grow and produce at low pH. The accumulation of fermentation end-products of the sludge leads to a fall in pH which introduce sufficient stress for hydrogen production. Generally, organic acids presented in the residual medium requires an appropriate disposal. Therefore, a utilization of these end-products for hydrogen production would increase the economic potential of hydrogen producing process by improvement of the hydrogen yield and reduction of the final waste COD.

Fig. 3 shows that the effluent from dark fermentation by the anaerobic sludge provided sufficient amount of organic acids as intermediates for photo-fermentation by *R. rubrum*. The main acids produced 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 i.e., 300 mL (Fig. 4) was obtained indicating that a sequential addition of anaerobic sludge and *R. rubrum* resulted in superior hydrogen production. A 200% increased in hydrogen yield was observed in sequential two-stage fermentation at pH 7, 30°C comparing to co-culture anaerobic sludge and *R. rubrum* in single stage at the same condition. Kim *et al.* [43] performed a two stage reaction with *C. butyricum* and *R. sphaeroides* and achieved an overall conversion of 1.64 mol hydrogen per mol glucose. Furthermore, Kataoka *et al.* [44] predicted that a two stage system for co-operative hydrogen production by *C. butyricum* and photosynthetic bacteria could achieved an overall yield of 5.6 mol hydrogen per mol glucose. Apparently, a sequential addition of anaerobic sludge and *R. rubrum* has certain advantages over single stage dark-fermentation and co-culture of anaerobic sludge and *R. rubrum* process. However, the optimum media composition and environmental conditions for the two

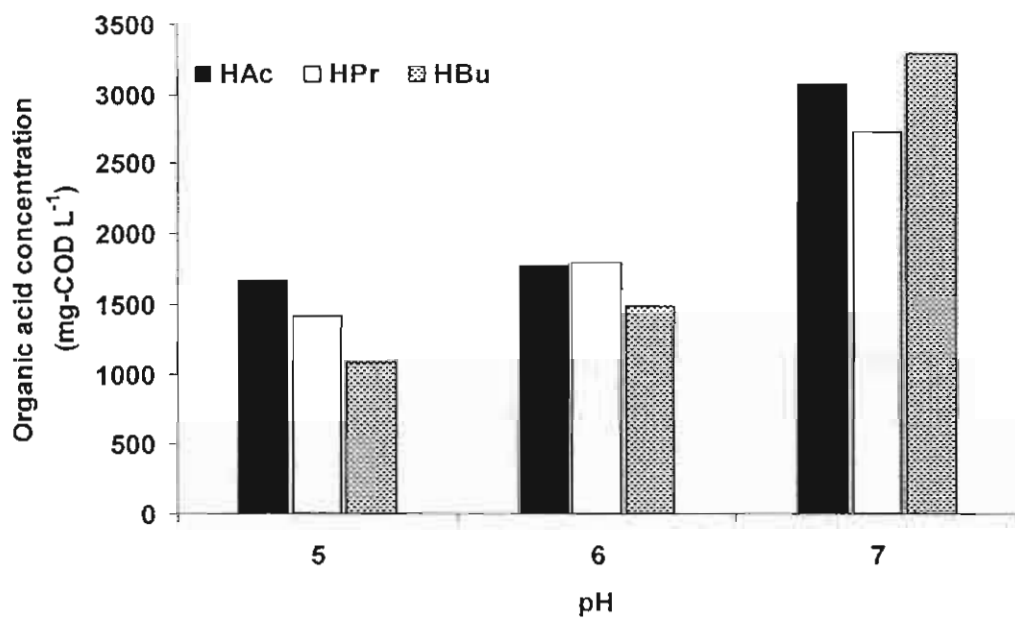


Fig. 3. Concentration of organic acids produced by a sequential addition of anaerobic sludge and *R. rubrum*.

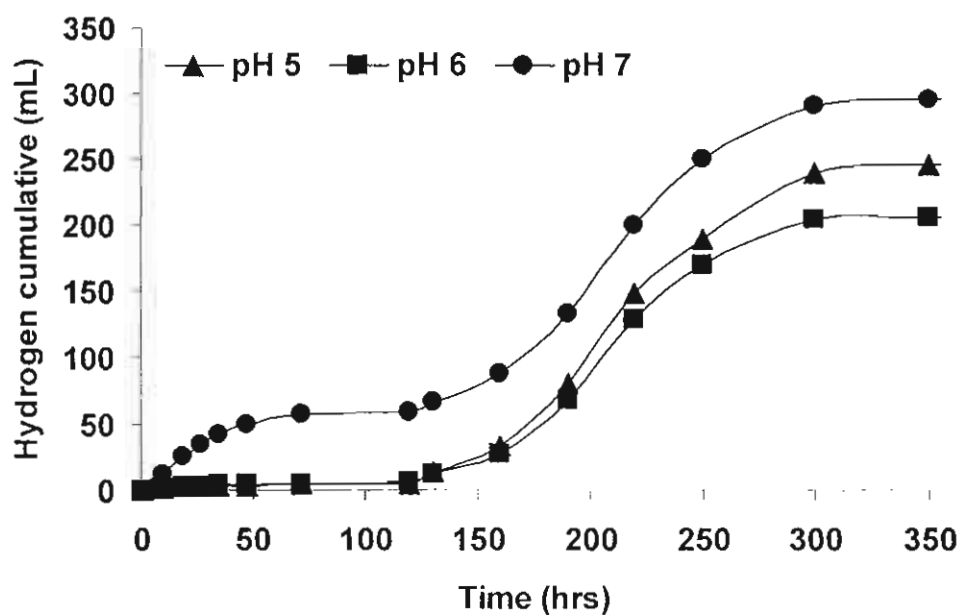


Fig 4. Hydrogen cumulative by a sequential addition of anaerobic sludge and *R. rubrum*.4.

microbial components of the process should be well controlled. Moreover, ammonia concentration in the effluent of the anaerobic fermentation should be below the inhibitory

level for the photosynthetic bacteria. Neutralization of dark fermentation effluents is therefore required prior to photo-fermentation in order to adjust the organic acid concentration and the initial pH to 7 for the optimal performance of photosynthetic bacteria [21, 22].

## Conclusions

In this research we have found that:

1) Maximum hydrogen production in batch culture of anaerobic sludge was achieved at 55 °C and initial pH 5.0 with a specific hydrogen production of 1,511 mL H<sub>2</sub>/g-VSS and a hydrogen yield of 251 mL/g-COD.

2) Results from hydrogen production by co-culture showed that the presence of *R. rubrum* improved the specific hydrogen production by 1.7 folds and the hydrogen yield by 1.7 folds when compared to the use of anaerobic sludge alone at the same conditions of 30°C and initial pH 7.0.

3) Superior results were obtained when a sequential addition of anaerobic sludge and *R. rubrum* was used to ferment hydrogen from cassava starch manufacturing wastewater. Hydrogen yield in terms of COD-H<sub>2</sub>/COD<sub>input</sub> of 0.28 and the cumulative hydrogen of 300 mL were obtained at 30°C and initial pH 7.0.

4) Our results suggested that cassava starch manufacturing wastewater is one of the potential sources of renewable biomass to produce hydrogen.

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## PART II

### **Bio-hydrogen Production from Cassava Wastewater by Upflow Anaerobic Sludge Blanket Reactor**

A paper to be submitted to International Journal of Hydrogen Energy

#### ABSTRACT

Sustainable hydrogen production from cassava wastewater was conducted in Upflow Anaerobic Sludge Blanket (UASB) reactor. Inoculum used to form UASB granule was anaerobic sludge obtained from the alcoholic wastewater treatment plant. Prior to granule formation, anaerobic sludge was heat treated in boiling water for 30 minutes. A pH of the substrate in the UASB reactor was maintained at pH 6-6.5 by adding  $\text{NaHCO}_3$ . Heat shock treatment and pH control were conducted to inhibit methanogenic activity. The 9.4-L UASB reactor was operated for 7 months at 5 different hydraulic retention time (HRT) i.e., 24, 18, 12, 8.4 and 4.8 h with concentrations of cassava wastewater and inoculum approximately 10,000 mg COD/L and 10,000 mg/L, respectively. The biomass concentration in the bed zone of the UASB reactor was increased throughout the experiment suggesting that hydrogen producing granulated sludge were developed. A shift in HRT from 24 h to 12 h appeared to enhance hydrogen production rate. When HRT was shortened from 12 h to 4.8 h the percentage of hydrogen produced decreased from 46% to 10%. Peak of the hydrogen yield of 46 mL  $\text{H}_2$ /g COD and the hydrogen production rate of 16.1 L/d were obtained at HRT 12 h. These results indicated that HRT 12 h was an optimum HRT in producing hydrogen from cassava wastewater. An average granular sludge at HRT 12 h was a light-grey in color and was 0.14 mm in diameter. Each gram of biomass produced 0.89 mL  $\text{H}_2$ /day with gas evolved mixture of 46% hydrogen, 40% carbon dioxide and less than 2% methane. The effluent volatile suspended solid, the endogenous decay coefficient ( $K_d$ ) and yield coefficient ( $Y_g$ ) of hydrogen producer granules were 235 mg/L and 0.64 /day and 0.93 g VSS/g COD, respectively. During the efficient hydrogen production stage, a major soluble metabolite was butyric acid, followed by acetic acid and propionic acid. Analysis of microbial by DGGE in

granulated sludge indicated that dominant species found in each band on the DGGE profile at every HRT were *Megasphaera elsdenii* and *Megasphaera hongkongensis*.

**Keywords:** bio-hydrogen, cassava wastewater, DGGE, microbial community, UASB

## INTRODUCTION

Hydrogen is considered to be an ideal and clean source of energy. Among many processes of hydrogen production, microbial hydrogen production has been extensively investigated due to its energy-saving process (Kumer and Das, 2000). Hydrogen production from waste using microorganism is attractive because lost energy can be recovered from wastes derived from renewable resources. (Lee et al., 2002). Microbial hydrogen production is classified into two categories (Yokoi et al., 1995). One is hydrogen production by photosynthetic microorganisms such as algae or photosynthetic bacteria. The other is hydrogen production by fermentative hydrogen-producing microorganisms such as facultative anaerobes and obligate anaerobes (Yokoi et al., 1995). Fermentative hydrogen-producing microorganisms have an advantage over photosynthetic microorganisms since fermentative hydrogen can evolved in a reactor continuously in the absent of light (Yokoi et al., 1995). Thus, dark fermentation has great potential in practical applications and seems to be a promising way for sustainable H<sub>2</sub> production (Levin et al., 2004).

Cassava starch production is a great extended industry in Thailand. The process to obtain starch from cassava root pollutes a huge volume of wastewater with high Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) which cause many environmental pollution (Richard and David, 2004). In addition, cassava wastewater contains intermediates for producing hydrogen i.e., acetic, propionic, and butyric acids of 415.78, 565.95 and 863.50 mg/L, respectively. (Polprasert, Rukvichitkul, 2003) In these instances, cassava wastewater can be a good candidate for bio-hydrogen production. Therefore, it is of our interest to recover energy from high organic content in the cassava wastewater before discharging it to the environment.

Anaerobic fermentation was reported to be an effective process for treatment and energy recovering from high-strength wastewater (Yu et al., 2002). Anaerobically hydrogen fermentation could be conducted in either batch (Yokoi et al., 1995; Kumar, Das 2000),

repeated batch (Chin et al., 2003) or continuous process (Chang, Lin, 2004). Continuous hydrogen production is the most effective technique because large volume of hydrogen could be produced continuously for a long period of operation time without inoculum preparation step (Chang, Lin, 2004). Upflow Anaerobic Sludge Blanket (UASB) process has been widely applied to the treatment of industrial wastewater with high organic concentration such as food and beverage industries (Lettinga et al., 1997, Lettinga et al., 1980). It is an extensively applied in continuous anaerobic treatment system with the advantage that high COD or BOD removal efficiency could be obtained at the short Hydraulic Retention Time (HRT) (Chang, Lin, 2004). Hydrogen production from various kinds of substrate by UASB reactor had been reported (Chang, Lin, 2004; Han, Shin, 2004; Yang et al., 2006). However, there is no information available on production of hydrogen from cassava wastewater by UASB process.

Therefore, this research aimed to develop the UASB process for sustainable hydrogen production from cassava wastewater by granulated sludge developed. The HRT was varied to be 24, 18, 12, 8.4 and 4.8 h in order to determine the optimum HRT for successive hydrogen production from cassava wastewater.

## **MATERIALS AND METHODS**

### **Wastewater**

Cassava starch manufacturing wastewater was obtained from Asia Modify Starch Factory, Kalasin Province, Thailand. The pH value of the wastewater was  $5.02 \pm 0.35$  and the total COD was  $22,600 \pm 2,750$  mg COD/L. Other compositions were shown in Table 1. After removal of suspended solids by simple gravity settling, cassava wastewater was used as a feed at the concentration of 10,000-14,000 mg COD/L. The wastewater was added with 6.7 g/L of  $\text{NH}_4\text{Cl}$  as nitrogen source and 0.8 g/L of  $\text{K}_2\text{HPO}_4$  as phosphorous source. In addition, KCl 0.0075 g/L,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  0.0081 g/L,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.0025 g/L,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  0.0042 g/L,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  0.00018 g/L and  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  0.015 g/L were added as trace elements for the anaerobic microorganisms.

Table 1 Compositions of cassava wastewater from Asia Modify Starch Factory. Data are given as mean $\pm$ SD, n = 3

Parameter	mg/L
COD	22,600 $\pm$ 2,750
Nitrogen	258 $\pm$ 92
Phosphorus	54 $\pm$ 24
Total suspended solid	1,193 $\pm$ 230
Volatile suspended solid	1,300 $\pm$ 316
Acetic acid	191 $\pm$ 48
Propionic acid	16 $\pm$ 3
Butyric acid	63 $\pm$ 17
pH	5.02 $\pm$ 0.35

### Inoculum

Anaerobic sludge was obtained from the alcoholic factory, Khon Kaen, Thailand. It was collected from a final sedimentation tank and then filtered through a 1,000  $\mu$ m mesh screened to separate out the large particulate matters. The sludge had the pH, total solids (TS) and volatile suspended solids (VSS) of 6.4, 23,500 mg/L and 19,400 mg/L, respectively. Sludge was heat-treated at 100°C for 30 min in order to inhibit the methanogenic bacteria prior the usage as inocula for hydrogen production in the UASB process.

### Reactor Design and Experimental Procedure

The 9.4-L UASB reactor was operated at the ambient temperature and pH was controlled to be in the range of 6.0-6.5 by adding NaHCO<sub>3</sub> at a concentration of 2.4 g/L as the buffer. Figure 1 illustrated the schematic diagram of UASB reactor used in this study. Working volume, interior diameter and height of this UASB reactor were 9.4 L, 9.5 cm, and 630 cm, respectively. The sampling ports were designed at the interval of 10 cm along the reactor tank. An operation of UASB was started by filling the reactor with 5 L of 19,400 mg VSS/L of the heat-treated (resulted in approximately 10,000 mg VSS/L sludge). Cassava wastewater was fed up gradient continuously through inoculated heat-treatment sludge in the reactor with the HRT of 24 h (0.4 L/h) for 62 days to allow granules formation. After the

granules were developed, the reactor was operated at HRT 24 to reach a steady state condition judged from stable values of COD present in the UASB reactor. After reached the steady state at HRT 24, the HRT reduction was conducted in a stepwise reduction through 18 (an influent velocity of 0.5 L/h), 12 (0.8 L/h), 8.4 (1.0 L/h) and 4.8 (2.0 L/h) hrs, respectively.

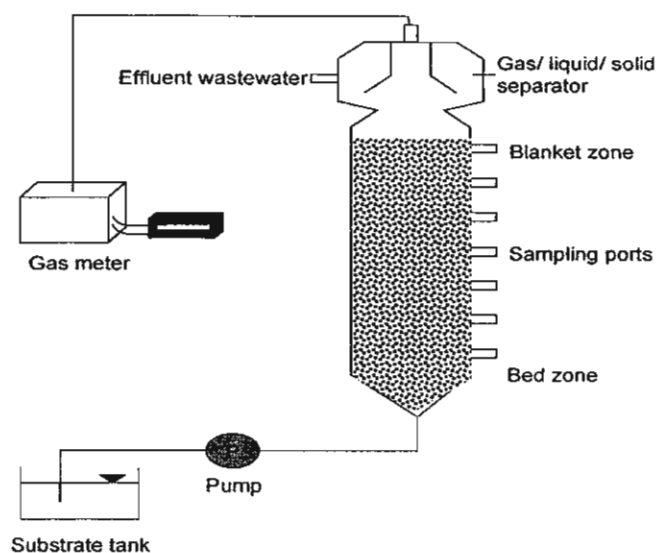


Figure 1 Schematic description of the UASB reactor for continuous hydrogen production

### Monitoring

The effluent and produced gas from the UASB reactor were sampled once a day to determine COD, volatile fatty acid (VFA), total suspended solid (TSS) and volatile suspended solid (VSS) concentration, gas production and gas compositions. Biomass was periodically taken from the sampling ports at the bed zone and blanket zone of the reactor to determine the VSS concentration according to the Standard Methods of APHA (1995). The granulated sludge were sampled from the reactor after the operation of UASB process at each HRT when the steady state was obtained in order to analyze the microbial community in the granulated sludge.

## Analytical method

### Gas analysis

The volume of biogas production was measured by gas meter and the analysis of biogas composition was performed using a gas chromatograph (GC) (Model 2014, SHIMADZU, Japan) equipped with a thermal conductivity detector (TCD). A 2 m x 2.5 mm stainless-steel column packed with 60/80 mesh Unibead C was used to analyze the content of hydrogen, nitrogen, methane and carbon dioxide in the biogas produced. Helium was used as the carrier gas at a flow rate of 25 ml/min. The temperature of injector, detector and column were 150, 150 and 145°C, respectively.

### Volatile Fatty Acid (VFA) Analysis

Concentrations of acetic, propionic and butyric acids were determined by a gas chromatography (Model 14B, SHIMADZU, Japan) equipped with a flame ionization detector (FID) and a 3 m x 3.2 mm glass column packed with 30/60 mesh Cabowax 20M. Injector, detector and column temperatures were at 200, 250 and 180°C, respectively. Nitrogen, hydrogen and pressured air were used as carrier gases with flow rate of 80, 70 and 500 mL/min, respectively.

### Kinetic constants

Kinetic constants of biological hydrogen production process from the UASB reactor were calculated by fitting to a following equation (Bitton, 1994).

$$V = \frac{1}{Y_g} D + \frac{K_d}{Y_g} \quad (1)$$

Where V is specific substrate utilization rate (mg COD/mg.day). D is dilution rate (/HRT).  $Y_g$  is yield coefficient expressing the cell mass produced per unit substrate.  $K_d$  is the endogenous decay coefficient (/day). The kinetic values for  $Y_g$  and  $K_d$  can be determined using the least-squares method to plot V vs. D according to Eq. (1).

### **Analysis of Microbial Community in Granulated Sludge**

The granulated sludges were sampled at each HRT (24, 18, 12, 8.4 and 4.8 h) for microbial analysis. Granules were firstly centrifuged at 14,000g for 30 sec to remove the fermentative broth. The granular pellets were then washed with a pH 7.4 phosphate buffer before centrifugation at 14,000g for 30 sec to remove washing buffer. The washed granules were then sonicated for 10 sec in ice box. The total community DNA from the granulated sludge was extracted on beading method with Fast DNA Spin kit of soil, Bio 101 (Biospec Products, Bartlesville, USA), followed by PCR amplification using the primer set of 357F-GC (5'-CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3') and 518 R (5'-GTATTACCGCGGCTGCTGG-3') with an annealing temperature of 72 °C in automated thermal cycle (Biorad, USA). The PCR amplified products were then screened using DGGE to investigate the microbial population variations under different HRT. A 40% acrylamide/Bis solution was used to cast a gel with denaturant gradients ranging 30-70%. Electrophoresis was conducted in a 1xTAE buffer solution at 130 V and 60°C for 5 hr. The 16S rDNA bands on the gel were then stained with ethidium bromide. The repeat PCR of DNA in presenting band was conducted 5 times in order to purify the DNA. The obtained PCR products were sequenced by Biomolecular Analysis Service Unit, Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Thailand. The nearly full length sequences were compared with the reference microorganisms available in the GenBank by BLAST search ([www.NCBI.com](http://www.NCBI.com)). The DNA sequences obtained and their closest 16S rDNA sequences of reference microorganisms retrieved from the GenBank were aligned and checked manually using the BioEdit. (Hall T, 2005)

## **3. RESULTS AND DISCUSSION**

### **Start up**

The UASB reactor was continuously fed with cassava wastewater after removal of the suspended solid by gravity settling. In order to inhibit methanogenic bacteria, the pH of reactor was maintained at 6.0-6.5 under the ambient temperature. At a HRT of 24 h, it was required 62 days to achieve constant gas production and granulated sludges were developed.

The granulation was not obvious during the start up period but the size slowly increased in the reactor afterward. After 60 days of reactor operation, the granule size of 0.12 mm (in average, n=100) was obviously predominant in the bed zone of reactor. The biomass concentration in the bed zone of UASB reactor increased from initial value of 10,000 mg VSS/L to 12,000 mg VSS/L. At day 119, the average granule diameter increased to 0.14 mm (in average, n=100) as the operated reactor progressed. The average granular diameter peak was 0.28 mm at day 157 (HRT, 8.4 h). Our granulated sludge were smaller than the previous report as by Fang et al. (2002) who found that a typical mature granule of hydrogen producer was 1.6 mm in diameter. We compared size of our granulated sludge of hydrogen producer and hydrogen producer granules developed by Fang et al., (2002) (1.6 mm) to methane producer granules and found that the size of hydrogen producer granules were smaller than methane producer granules (2.6 mm) (Lin and Chen, 2001).

The gas production and hydrogen gas content from the reactor at the start up periods were low (3 L/day and 4%, respectively). However, after reaching steady state, the gas production and hydrogen gas content were gradually increased to be 11 L/day and 19%, respectively. It was found that a great amount of intermediate acidic production i.e., acetic (1,733 mg COD/L), butyric (2,334 mg COD/L) and propionic (952 mg COD/L) acids were produced in the reactor by granulated sludge.

### **Reactor Operation**

After granule formation, the UASB reactor was conducted at the HRT of 24 h until reaching the steady-state (29 days). The hydrogen bio-production was started at day 63 after starting up the process. Then, the HRT was stepwise reduced to 18, 12, 8.4 and 4.8 h through 19, 24, 24 and 20 days, respectively. The COD removal efficiency was observed to be over 60% at the HRT of 24 h. When HRT was less than 24 h, COD removal efficiency decreased remarkably to less than 60% (data not shown).

The results of the UASB operation at each HRT were shown in Table 2. The hydrogen production observed at the steady state of UASB operation was found to vary with the changing of HRT. The hydrogen generation rate increased when the HRT decreased from 24 h to 18 and 12 h, respectively (Figure 2). Results indicated that at the HRT of 12 h the hydrogen production in UASB process was effectively evolved with the highest average

Table 2 General Data in the UASB Reactor under Steady-state Conditions at each HRT

HRT (h)	Operation time (day)	OLR <sup>a</sup> (g COD /L.day)	Hydrogen production rate (L/d)	Peak of		Specific H <sub>2</sub> production (mL H <sub>2</sub> / day.g biomass)	VFA (mg COD/L)	VFA compositions			
				H <sub>2</sub> Yield (mL H <sub>2</sub> / g COD)	H <sub>2</sub>			HAc (%)	HPr (%)	HBu (%)	HBu/HAc ratios
24	29	10	6.7	36		0.56	4,611	32	18	50	1.56
18	19	13.3	7.5	37		0.44	4,741	33	24	43	1.30
12	24	20	16.1	46		0.89	4,708	34	21	45	1.32
8.4	24	28.6	5.9	21		0.33	4,604	28	18	54	1.93
4.8	20	48	3.3	10		0.17	4,506	23	20	57	2.48

<sup>a</sup>Organic Loading Rate

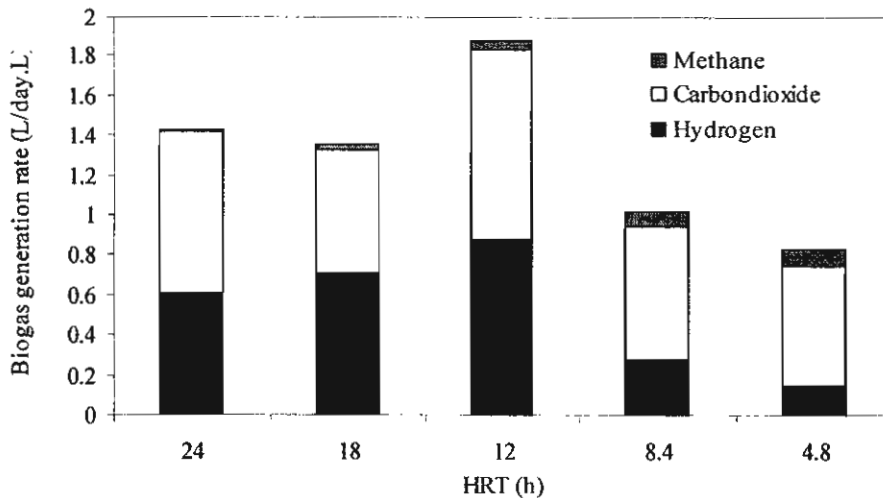


Figure 2 Biogas generate at different steady state of UASB

steady state hydrogen production rate of 16.1 L/day. Produced biogas contained of 46 % hydrogen, 40% carbon dioxide and 2% methane indicating that hydrogen producing microorganism was dominant in the UASB reactor operated at this HRT. At low HRT, hydrogen producers were dominant and ineffective hydrogen production microflora such as methanogens were sorted out (Lin and Chen, 2001). Compared with other UASB for hydrogen production, the maximum HRT were found to be various. Chang and Lin (2004) reported that a maximum rate of hydrogen production from sucrose by heat treated sludge was 270.6 mmol H<sub>2</sub>/L.day occurred at HRT 8 h. Yu et al. (2002) found that the maximum specific rate of hydrogen by a mixed bacterial flora in a thermophilic (55°C) upflow reactor, using rice winery wastewater as a substrate was 9.33 L H<sub>2</sub>/g VSS.day at an HRT 2 h and pH 5.5. Ueno et al.(1996) evaluated the effect of HRT on the production hydrogen from sugar industry wastewater by anaerobic microflora in chemostat culture and reported that a maximum hydrogen yield of 14 mmol H<sub>2</sub>/g carbohydrate removed (2.6 mol H<sub>2</sub>/mol hexose) was obtained at an HRT 12 h. Hydrogen production from glucose by acclimated sludge in a completed mixed reactor was study with the variation of HRT to be 12.5, 10, 8, 6 and 4 h (Majizat et al., 2000). Results indicated that the most effective HRT for hydrogen production was 12.5 h, in which hydrogen could be produced at the highest concentration of 71% following by HRT 4 h (67%) and 6 h (61%), respectively.

Kinetic parameters of biological hydrogen production process were determined using equation (1). Endogenous decay coefficient ( $K_d$ ) and Growth yield coefficient ( $Y_g$ ) obtained were 0.64 /day and 0.93 g VSS/g COD, respectively. These data will be useful when an effective UASB process for hydrogen productions are designed (Chang and Lin, 2004). Our  $K_d$  and  $Y_g$  were much higher than that in a hydrogen producing UASB reactor fed on glucose (0.12-0.18 g VSS/g COD) (Fang and Liu, 2002). In addition, our  $K_d$  and  $Y_g$  were higher than those values for mixed methanogenic granule culture (0.126g VSS/g COD, Guiot et al., 1992) and  $K_d$  for methanogenic fed on volatile fatty acid (0.1 /day, Lin et al., 1989).

At the HRT of 4.8 and 8.4 h, the steady state of hydrogen generation rate was found to be less than 0.4 L/d.L (Figure 2). This may due to the wash out of hydrogen producers from the system indicated by high effluent SS discharge rate of 32,806 mg/day (HRT 4.8 h) and 14,397 mg/day (HRT 8.4 h) which were higher than that of HRT 12 h (7,896 mg/day), 18 h (4,560 mg/day) and 24 h (1,847 mg/day), respectively

An increase of  $CO_2$  and  $CH_4$  concentration in biogas generated at these two HRTs (Figure 2) implying the presence of methanogenic bacteria in the process, in which they could survive and adapt themselves to produce methane at low HRTs (4.8, 8.4 h). Pre-heated treatment of sludge at 100 °C for 30 min and the control pH of 6.0-6.5 could not inhibit methanogenic bacteria completely. Further study on an effective inhibition of methanogenic community should be conducted to resolve this issue.

$CO_2$  production decreased when the organic loading rate increased or HRT decreased. A similar result was reported by Chang et al. (2002) in which an operation at high carbon substrate loading rate or low HRT led to a conversion of starch to  $CO_2$  by bacterial populations that were inefficient for hydrogen production.

Anaerobic hydrogen production was always accompanied with VFA production (Figure 3). The VFA profiles had been successfully used as indicators for monitoring hydrogen production (Chen et al., 2002). The main intermediate product was butyric acid with a mean concentration of 50%, acetic acid was in the range 28-34% and propionic acid was in the range 18-24%. The highest amount of acetic acid was produced at HRT 12 hr (Table 2). Overall average concentrations of major liquid products i.e., butyric, acetic and propionic acids were 1,449-2,960, 1,228-2,290, 577-1,420 mg COD/L, respectively (Figure 3). HBU/HAC ratio was calculated to evaluate hydrogen production. The relatively low

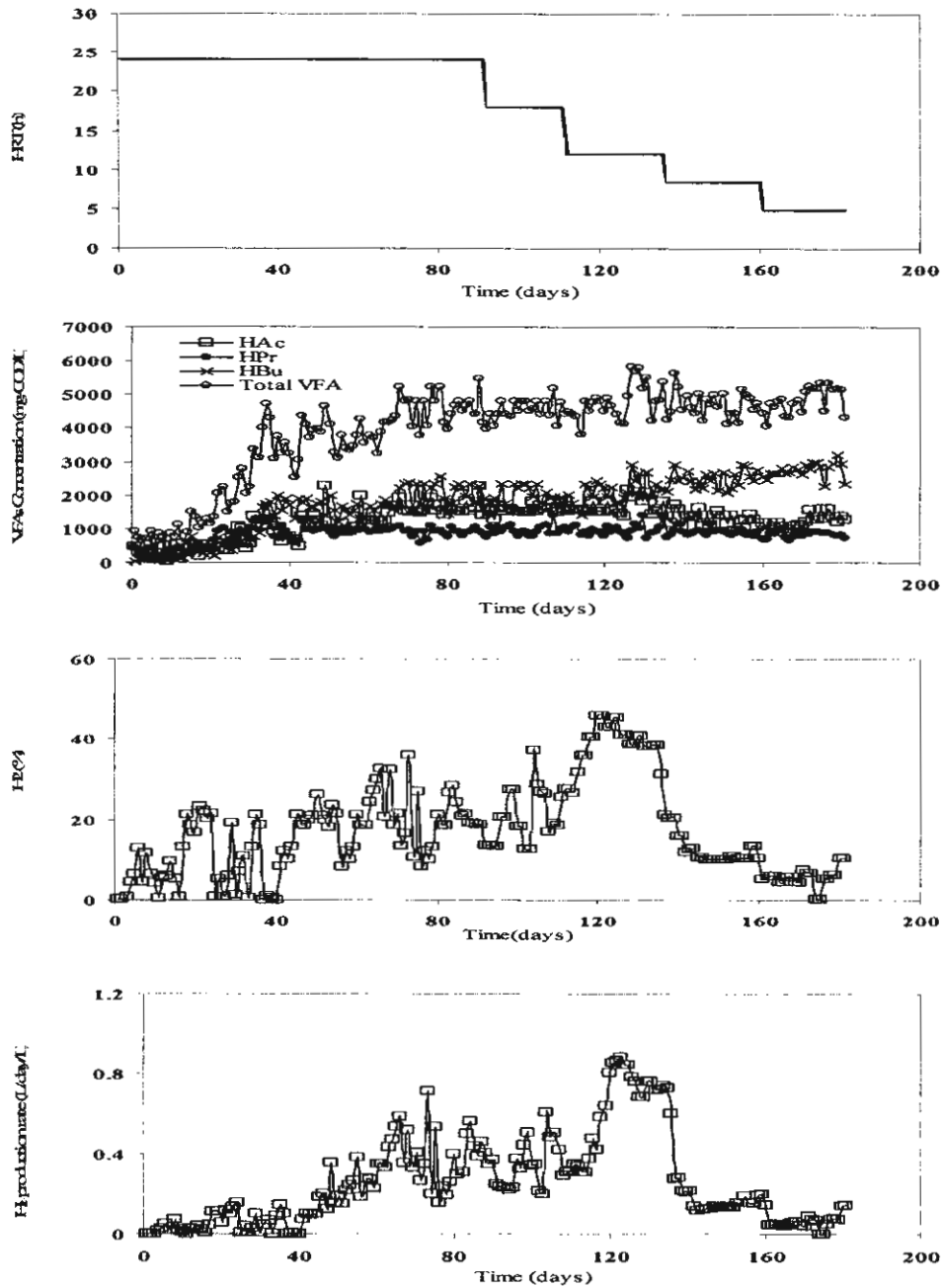
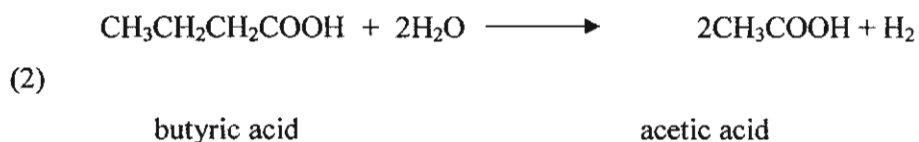


Figure 3 Time course of variations in (a) HRT, (b) VFA concentration, (c) hydrogen content and (d) hydrogen production rate.

HBu/HAc ratio of 1.32 was observed at the HRT 12 (Table 2) indicating a good transformation of butyric acid to acetic acid resulting in a high amount of hydrogen production as described in equation (2) (Fang , Zhang, 2005).



Biomass concentrations in the bed zone were varied from 12,100 mg VSS/L or 12 g VSS/L (HRT of 24 h) to 18,956 mg VSS/L or approximately 19 g VSS/L (HRT of 4.8 h) (Table 3) which were greater than that of the blanket zone throughout the experiment while the biomass concentration in bed zone were found to decrease. However, a biomass concentration of UASB was higher when compared with the other reactor system. For example, biomass concentrations in anaerobic continuous flow stirred tank reactor feeding on sucrose was reported to be 0.7-2.3 g VSS/L (Lin and Chen, 2001). A high level of biomass concentration is one of the expected characteristics of a UASB reactor (Chang and Lin, 2004).

Table 3 Biomass concentration

HRT (h)	Biomass Concentration (mg VSS/L)		
	Bed Zone	Blanket Zone	Effluent
24	12,124	736	162
18	17,359	657	213
12	18,367	331	235
8.4	18,293	287	429
4.8	18,956	284	565

At an optimum HRT for hydrogen production i.e., 12 h, each gram of biomass in the UASB process produced 0.89 mL H<sub>2</sub>/day and the effluent VSS rate of 4,418 mg/day. Periodically discharging sludge was a possible means of dealing with sludge production from a biological system. To evaluate the performance of an UASB reactor, it is important to know whether the reactor could be operated under conditions where the effluent settleable solid concentration is minimized by periodically discharging excess sludge before the maximum sludge holdup attained (Haandel and Lettinga, 1994).

The VSS to TSS concentration ratio (VSS/TSS ratio) in a liquid substrate fed to the biological reactor over HRT was illustrated in Figure 4. This value represented

the organic component in the biomass sludge and the values were in the range of 0.73-0.82 (Figure 4). VSS is the concentration of biomass in the fermentative broth while TSS is the concentration of total suspended solid in the fermentative broth. Thus, our VSS/TSS ratios in this study closed to 1.0 indicated that high amount of biomass was developed in the reactor. High biomass developed suggested a successful granule formation by UASB reactor.

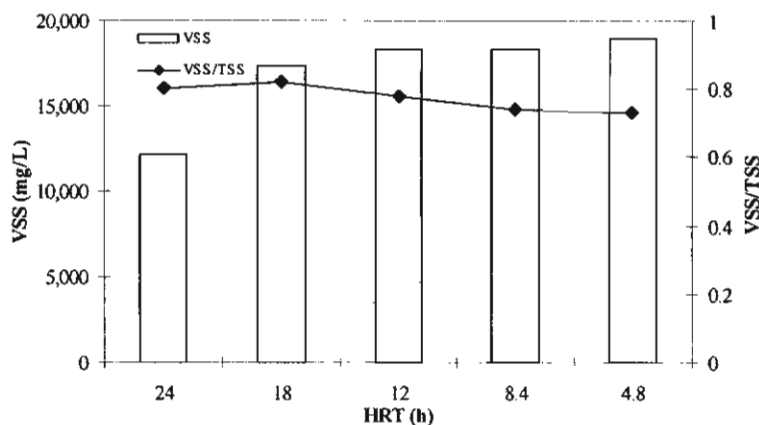


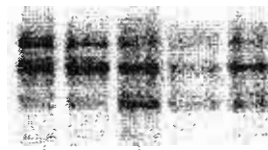
Figure 4 VSS and VSS/TSS obtained under steady-state conditions at each HRT.

### Microbiology

Sludge granulation in the UASB reactor was obviously observed during the start up period after 60 days of reactor operation. At that time, small visible of 0.12 mm in diameter granules was observed in the bed zone. At an optimum HRT (12 h), granulated sludge was found to be in a gray-white color with an average mature diameter of 0.14 mm.

Figure 5 illustrated the DGGE profiles of the 16S rDNA gene fragment amplified from the granule sampled from HRT 24, 18, 12, 8.4 and 4.8 h, respectively. Each band on the DGGE profile represented a gene fragment of unique 16S rDNA sequences, indicating a specific species in the microbial community. The staining intensity of a band represents the relative abundance of the corresponding microbial species. The DGGE profiles clearly showed that the microbial community changed with HRT (Figure 5). The more number of bands was observed in the granule obtained from the UASB process operated at higher HRT (Figure 5). All of appeared bands were identified for microbial species. Dominant species found in bands at every HRT were *Megasphaera elsdenii* and *Megasphaera hominis* which had similarity

percentages of 100% and 99%, respectively. These two species were reported as the hydrogen producing bacteria in the genus of Clostridia which were well known as hydrogen producer.



24 18 12 8.4 4.8

Figure 5 DGGE Profile for Hydrogen Producing Communities at HRT 24, 18, 12, 8.4 and 4.8 h.

#### **Acknowledgements**

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#### **4. CONCLUSIONS**

Conclusions drawn from this study are as follows:

1. UASB process was effectively applied in hydrogen production from cassava wastewater by heat treated anaerobic sludge as inoculum.

2. The biomass concentration in the bed zone of the UASB reactor increased throughout the experiment suggested that the hydrogen producer granular were developed.
3. HRT 12 h was the most optimum HRT for hydrogen production from cassava wastewater indicated by the highest hydrogen yield and total hydrogen production of 46 mL H<sub>2</sub>/g COD and 16.1 L/day, respectively, and relatively low the washed out sludge discharge rate of 7,896 mg/day. Each gram of biomass produced 0.89 mL H<sub>2</sub>/day with gas evolved mixture of 46% hydrogen, 40% carbon dioxide and less than 2% methane.
4. The endogenous decay coefficient (K<sub>d</sub>), and growth yield coefficient (Y<sub>g</sub>), of hydrogen producer granules of the UASB process were 0.64 /day, 0.93 g VSS/g COD.
5. Analysis of microbial by DGGE in granulated sludge indicated that dominant species found in each band on the DGGE profile at every HRT were *Megasphaera elsdenii* and *Megasphaera honisis*.

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## OUTPUTS

The outputs from this research are two oral presentations in the International Conference and two manuscripts which one has already been submitted to the International Journal and another one is in preparation.

Oral presentations at the International Conference:

1. "Biohydrogen Production from Cassava Starch Manufacturing Wastewater" was presented in JGSSE and Kyoto University Joint International Conference on Sustainable Energy and Environment, 1-3 December 2004, Hilton Hua Hin Resort & Spa, Hua Hin, Thailand. Vol. 1, p 319-324

Reungsang A., Sangyoka S., Imai T. and Chaiprasert P. 2004. Biohydrogen Production from Cassava Starch Manufacturing Wastewater. Proceeding of JGSSE and Kyoto University Joint International Conference on Sustainable Energy and Environment. 1-3 December 2004. Hilton Hua Hin Resort & Spa, Hua Hin, Thailand. Vol. 1, p 319-324

2. "Bio-hydrogen Production from Cassava Wastewater by Upflow Anaerobic Sludge Blanket Reactor" will be presented in International Conference on Environment 2006, 13-15 November 2006, Grand Plaza Parkroyal Resort, Batu Ferringhi Beach, 11100 Penang, Malaysia.

Manuscripts:

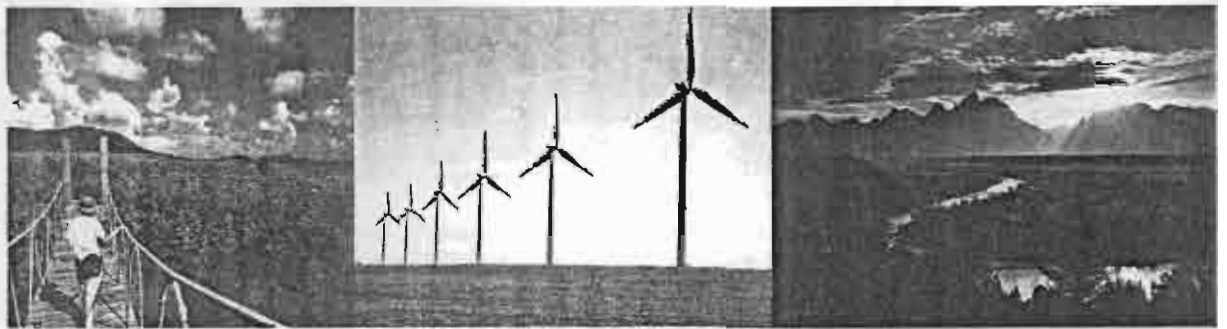
1. Biohydrogen Production from Cassava Starch Manufacturing Wastewater.  
A paper submitted to publish in the Biomass Bioenergy (Manuscript code 06-174)
2. Bio-hydrogen Production from Cassava Wastewater by Upflow Anaerobic Sludge Blanket Reactor.  
A paper to be submitted to International Journal of Hydrogen Energy

**APPENDIX**

JGSEE and Kyoto University  
Joint International Conference



# *Sustainable Energy and Environment*

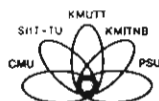


## **PROCEEDINGS**

1-3 December 2004

Hilton Hua Hin Resort & Spa  
Hua Hin, Thailand

Volume 1



# **JGSEE**

The Joint Graduate School of Energy and Environment



Kyoto University

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**Biohydrogen Production from Cassava Starch Manufacturing Wastewater**Alissara Reungsang<sup>1,2,\*</sup>, Suksaman Sangyoka<sup>1,2</sup>, Tsuyoshi Imai<sup>3</sup> and Pawinee Chairprasert<sup>4</sup><sup>1</sup>Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen, Thailand<sup>2</sup>Fermentation Research Center for Value Added Agricultural Products, Khon Kaen University, Khon Kaen, Thailand<sup>3</sup>Department of Civil and Environmental Engineering, Faculty of Engineering, Yamaguchi University, Yamaguchi 755-8611, Japan<sup>4</sup>Program of Biotechnology, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkok, Thailand

**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 °C and pH 5.0 with a specific hydrogen production of 429 mL H<sub>2</sub> g<sup>-1</sup>-VSS and a hydrogen yield of 71.3 mL g<sup>-1</sup>-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°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°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*.

**1. 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<sup>-1</sup> 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 photosynthetic 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.

**2. MATERIAL AND METHODS****2.1 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<sup>-1</sup>, respectively.

**2.2 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

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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<sup>-1</sup>, 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<sub>2</sub>HPO<sub>4</sub>, 0.5% MgSO<sub>4</sub>, 10% yeast extract, 2.01% DL-malic acid and 0.33% DL-glutamic acid.

### 2.3 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<sup>-1</sup> of 100:10:1 mg L<sup>-1</sup> by using NH<sub>4</sub>Cl and K<sub>2</sub>HPO<sub>4</sub> 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).

### 2.4 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<sup>-1</sup> (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.

### 2.5 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 20,000 mg L<sup>-1</sup> (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°C without a pH control under illumination at 6000 lx by the fluorescent lamp.

## 2.6 Analytical method

### 2.6.1 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 8APP, 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<sup>-1</sup>. The temperatures of injector, detector and column were 50, 50 and 60°C, respectively.

### 2.6.2 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°C, respectively. Nitrogen, hydrogen and pressured air were used as carrier gases with flow rate of 50, 60 and 500 mL min<sup>-1</sup>, respectively.

### 2.6.3 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<sup>-1</sup>),  $e$  is 2.71828 and  $\lambda$  is the lag time (hr) and  $t$  is time. The maximum specific hydrogen production rate (mLg<sup>-1</sup>-VSS.hr) was calculated by dividing  $R_m$  by the initial sludge VSS. The hydrogen yield or conversion efficient (mL g<sup>-1</sup>-COD wastewater) was calculated by dividing  $P$  by the g-COD waste water. The specific hydrogen production (mL H<sub>2</sub> g<sup>-1</sup>-VSS) was calculated by dividing  $P$  by g-VSS.

## 3. RESULTS AND DISCUSSION

### 3.1 Hydrogen production from cassava wastewater by anaerobic seed sludge

Mesophilic temperature (30 and 35°C) and thermophilic temperature (45 and 55°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°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 °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.



At thermophilic temperature (45 and 55°C) the HAc and HBU were major components (about 50% for each) except at pH 5, 55°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<sub>2</sub> and O<sub>2</sub>. *C. acetivum* can reduce CO<sub>2</sub> to acetate with H<sub>2</sub> 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°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<sup>7</sup> CFU mL<sup>-1</sup>. 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 (°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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> from cassava wastewater by anaerobic seed sludge was 18.01 at pH 5, 55°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 g<sup>-1</sup>-COD and 440 mL H<sub>2</sub> g<sup>-1</sup>-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°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 °C, when the P values were high the R<sub>m</sub> values were also high. Chen et al. [26] explained that this may due to the fact that sludge microorganisms possess a range of responsive capacities for different adverse circumstances resulted in a certain characteristic, such as P, R<sub>m</sub> and λ. The λ value was high at 55°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°C and pH 7.0 (Table 2). This indicated that *R. rubrum* was effective in producing hydrogen.

### 3.2 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°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 H<sub>2</sub> (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 be due to the fact that H<sub>2</sub> 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<sub>4</sub> and CO<sub>2</sub>. Prior to

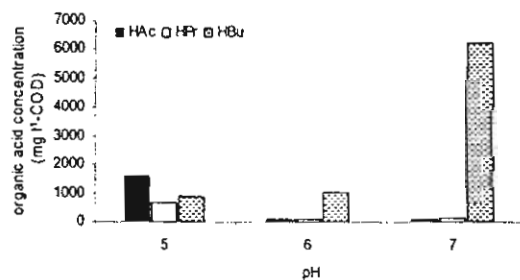
the methanogenic stage, hydrogen was obtained as an intermediate product and was utilized as it was produced by methanogenic archaea, acetogenic bacteria and sulfate reducing bacteria [15]. Therefore, if the hydrogen consuming bacteria can be inhibited, the more hydrogen produced will be obtained. The other reason that the volume of hydrogen evolved was low (Fig.2) may be 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 mg l<sup>-1</sup>, by anaerobic seed sludge

Initial pH	T (°C)	λ (h)	R <sub>m</sub> (mL h <sup>-1</sup> )	P (mL)	Maximum specific H <sub>2</sub> production rate (mL g <sup>-1</sup> -VSS-h)	Specific hydrogen production (mL H <sub>2</sub> g <sup>-1</sup> -VSS)	Hydrogen yield (mL g <sup>-1</sup> COD)	R <sup>2</sup>
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

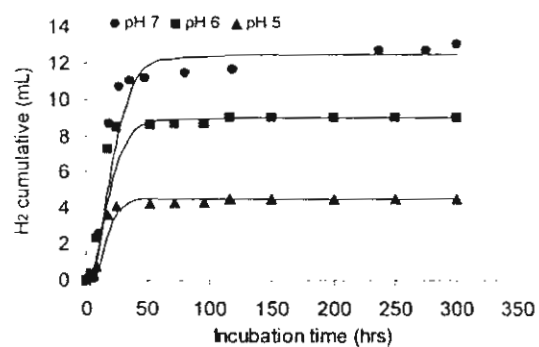
### 3.3 Hydrogen production from cassava wastewater by a two-step batch culture of anaerobic seed sludge and *R. rubrum*

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*.

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



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

**Table 3** Modified Gompertz equation parameters for hydrogen production from cassava wastewater, 10,000 mg L<sup>-1</sup>, by a mixed culture of anaerobic seed sludge and *R. rubrum*.

Initial pH	Temp (°C)	$\lambda$ (hr)	$R_m$ (mL hr <sup>-1</sup> )	$P$ (mL)	Maximum specific H <sub>2</sub> production rate (mL g <sup>-1</sup> -VSS-d)	Specific hydrogen production (mL H <sub>2</sub> g <sup>-1</sup> -VSS)	Hydrogen yield (mL g <sup>-1</sup> COD)	R <sup>2</sup>
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

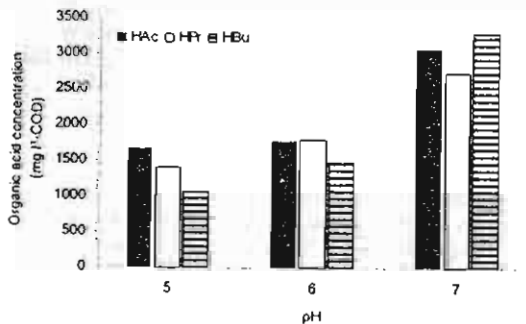
organic acids obtained in culture broth. We adjusted the pH of the culture broth from below 5 to pH 7 before adding *R. rubrum* into the culture broth. This was because there was no H<sub>2</sub> gas produced without pH adjusted (data not shown) suggesting *R. rubrum* cannot grow at low pH and the optimum 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.

#### 4. CONCLUSIONS

In this research we have found that:

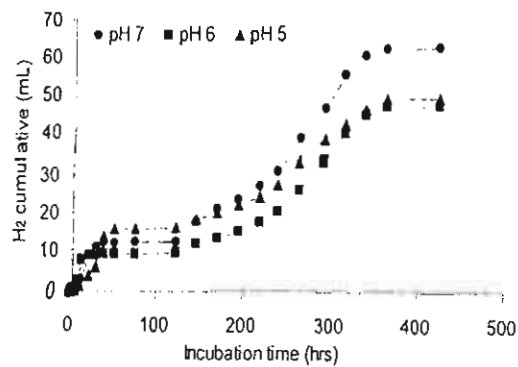
- 1) Maximum hydrogen production in batch culture of anaerobic seed sludge was achieved at 55 °C and pH 5.0 with a specific hydrogen production of 440 mL H<sub>2</sub>g<sup>-1</sup>-VSS and a hydrogen yield of 71 mL.g<sup>-1</sup>-COD.
- 2) Results from hydrogen production by the mixed culture showed 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 30°C 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°C and pH 7.0

4) Our results suggested that cassava wastewater is one 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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