CHAPTER VII
ENHANCEMENT OF BIOHYDROGEN PRODUCTION FROM SWEET SORGHUM SYRUP BY ANAEROBIC SEED SLUDGE IN ANAEROBIC SEQUENCING BATCH REACTOR BY NUTRIENT AND VITAMIN SUPPLEMENTATIONS

7.1 Introduction

Recently, numerous research have been devoted to converting the inexpensive waste biomass, e.g. agricultural and agro-industrial wastes, into bioenergy such as ethanol, biodiesel and biohydrogen (Wilkie et al., 2000; Tsai et al., 2007; Virie et al., 2007) in order to replace the depleting of petroleum-based energy. Hydrogen is the choice of interest due to its high energy content as well as its environmentally friendly characteristic i.e. after the combustion water is the sole end product (Das and Veziroglu, 2001). Biohydrogen production by microorganisms can be divided into two main categories: (i) by photosynthetic bacteria cultured under anaerobic or semi-anaerobic light conditions, and (ii) by fermentative bacteria via dark fermentation (Liu et al., 2011). Dark fermentation converts organic pollutants into hydrogen by dark fermentative bacteria in the absence of light, producing organic acids, mainly acetate and butyrate, and alcohols as by-products. Photo fermentation is able to convert acids and alcohols, which are the by-products of dark fermentation, into hydrogen by photosynthetic bacteria using light as energy source. Dark fermentation has a high production rate of hydrogen, but with a low hydrogen yield (HY), converting no more than 40% of the chemical energy in the organic pollutants into hydrogen (Li and Fang, 2007). However, the dark fermentation was reported to be more economically feasible than photo fermentation (Lay et al., 2010). Therefore, if the low HY from the dark fermentation process can be improved, the dark fermentation can be the potential process to produce hydrogen from waste biomass.

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Various improvement methods have been applied to improve the yield of hydrogen from dark fermentation. For example, the optimization of bioprocess parameters such as substrate concentration, hydraulic retention time (HRT) and organic loading rate (OLR) (Zhao et al., 2008; Saraphirom and Reungsang, 2010b, c), sparging with CO_2 or N_2 (Kim et al., 2006) and nutrient supplementation under thermophillic condition (O-Thong et al., 2007). Significant improvement of HY was reported by O-Thong et al. (2007) that the nutrient supplementation improved hydrogen production from palm oil mill effluent (POME) by thermophillic microflora from 1.6 mol H_2/mol hexose to 2.24 mol H_2/mol hexose. Nutrient such as nitrogen (peptone and yeast extract), phosphorus, vitamins and mineral salts were essential for the synthesis of macro- and micro-molecules in the cell (Li and Fang, 2007), resulting in the enhancement of the microbial degradation as well as the hydrogen production process (O-Thong et al., 2007).

Continuously biohydrogen production from the energy crop such as cassava (Luo et al., 2010), sugarcane (Pattra et al., 2010), corn (Arooj et al., 2008) and sweet sorghum (Saraphirom and Reungsang, 2010b) have been reported. However, using crop such as sugarcane, corn and cassava as the substrate for energy production has led to the conflicts of food crop or energy crop. Therefore, the search for a new energy plant has been carried out. Sweet sorghum (*Sorghum bicolor* var. Keller) has been received the attention as a potential alternative feedstock for energy and industry due to its high biomass yield and high sugar content (Jaisil, 2007). Fresh biomass and fresh stalk of sweet sorghum yield 8-11.5% and 9-14.5% of total sugar content, respectively (Bulawayo et al., 1996). The main sugars in the sweet sorghum syrup are sucrose, fructose and glucose which are good substrates for hydrogen production. However, previous reports showed that a low maximum HY of 0.86 mol H_2/mol glucose consumed was obtained from the sweet sorghum extract in the continuous stirred tank reactor (Antonopoulou et al., 2008). In addition, the low HY of 0.53-0.67 mol H_2/mol hexose were obtained when sweet sorghum syrup was used as the substrate by continuous fermentation in anaerobic sequencing batch reactor (ASBR) (Saraphirom and Reungsang, 2010b, c). The low HY obtained might be the results from the lack of nitrogen, iron and phosphorus content in the juice composition

(Laopaiboon et al., 2009). Thus, the supplementations of nutrients and vitamins are needed in order to improve the HY.

Therefore, this study attempted to improve a bio-hydrogen production from sweet sorghum syrup by anaerobic seed sludge in ASBR by nutrient and vitamin supplementations. Effect of nutrient and vitamin supplementations on microbial community was also analyzed using polymerase chain reaction-denatured gradient gel electrophoresis (PCR-DGGE) technique. It is hoped that the proposed approach would increase the HY from sweet sorghum syrup.

7.2 Materials and Methods

7.2.1 Seed sludge and inoculum

Anaerobic seed sludge was obtained from a full scale anaerobic digester of Up-flow Anaerobic Sludge Blanket (UASB) reactor of the brewery company in Khon Kaen, Thailand. The UASB is used to produce methane from the wastewater of beer production process. Prior to use, the anaerobic sludge was heated at 105 °C for 2 hr in drying oven (LDO-100E, Daihan Labtech Co., Ltd, Korea) to inactivate methanogenic bacteria and then cooled at room temperature in dessicator. The heat treated sludge was further used as seed inoculums in hydrogen production experiment.

7.2.2 Sweet sorghum syrup

Sweet sorghum (*Sorghum bicolor* var. Keller) was obtained from the field experiment of Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand. Sweet sorghum syrup was prepared by concentrating sweet sorghum juice by heating to evaporate the water. Initial composition of sweet sorghum syrup was analyzed and showed in Table 1. The syrup was diluted by distilled water to obtain 30 g/L total sugar as initial substrate concentration. After dilution, the syrup composition consisted of (all in mg/L); 8.7±1.5 ethanol, 26.3±2.7 acetic acid, and 8.5±1.6 butyric acid.

7.2.3 Nutrient and vitamin compositions

The nutrient solution used in the experiment was modified from the Endo nutrient formula (Lin and Lay, 2005) but without $FeSO_4.7H_2O$. Each liter of medium was supplemented with 10 mL of each nutrient stock solutions. The nutrients were separately prepared into four stocks. The stock solutions consisted of

(all in g/L) 524 NH_4HCO_3 (stock I); 125 K_2HPO_4 (stock II); 672 $NaHCO_3$ (stock III); 100 $MgCl_2.6H_2O$, 15 $MnSO_4.6H_2O$, 5 $CuSO_4.5H_2O$, 0.125 $CoCl_2.5H_2O$ (stock IV). The vitamin solution was modified from Su et al., (2009) and supplemented to the fermentation broth at 10 mL/L of medium. The vitamin solution comprised of (all in g/L); glutamic acid 0.01, ascorbic acid 0.025, riboflavin 0.025, citric acid monohydrate 0.02, *p*-aminobenzoic acid 0.01 and creatinine 0.025.

Table 1 Sweet sorghum syrup compositions.

Parameter	Value
Total COD (g/L)	566-570
Total solid (g/L)	295-300
Total sugar (g/L)	800-820
-Fructose (%w/v)	32.19
-Glucose (%w/v)	35.70
-Sucrose (%w/v)	32.58
Total volatile fatty acid and alcohol (g/L)	41-45
-Acetic acid (%w/v)	54.63
-Propionic acid (%w/v)	-
-Butyric acid (%w/v)	20.70
-Lactic acid (%w/v)	-
-Ethanol (%w/v)	20.73
-Butanol (%w/v)	-
-Acetone (%w/v)	-

7.2.4 Operational condition and monitoring

The reactor was made from "Acrylic" material with a total volume of 1.3 L (1 L liquid volume, 0.3 L gas holding capacity). Configuration of the reactor was shown in Figure 1. The ASBR was operated at room temperature (30±3 °C) in suspended mode using magnetic stirrer (Stuart heat-stir CB162, Keison International Ltd., USA). The feeding, decanting and settling of the ASBR were automatically controlled by digital time controller (TS-ET1, China). Two peristaltic pumps (Eyela roller pump RP-1000, Tokyo Rikakikai Co. Ltd., Japan) were used for transferring the

influent and effluent of the reactor. During the experiments, 2N NaOH solution was used to maintain pH within 5.0±0.1 using pH meter and controller (pH 190 series, Eutech Instruments, Singapore). Oxidation reduction potential (ORP) was monitored using the same model of pH meter.

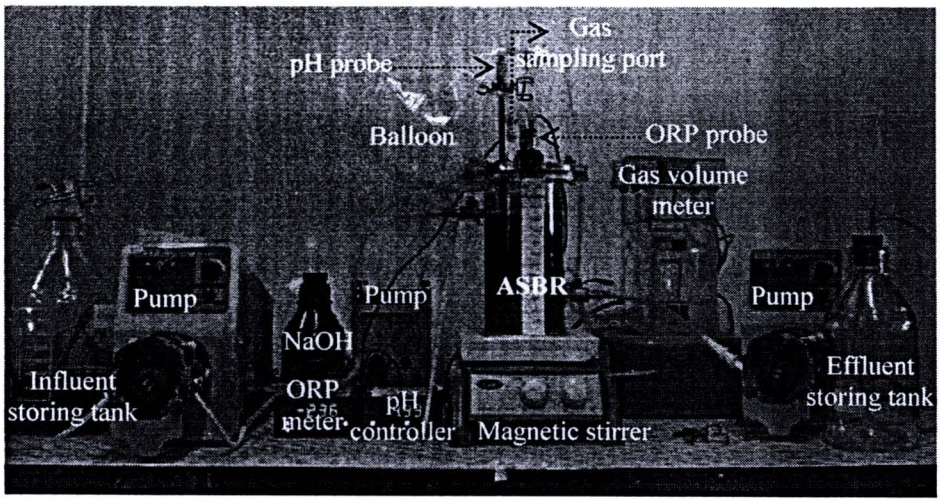

Figure 1 Anaerobic sequencing batch reactor configuration.

ASBR was started up by inoculating 100 mL of heat treated seed sludge (equivalent to 500 mg/L as measured by volatile suspended solid (VSS)) into the reactor containing 900 mL of enrichment medium that consisted of sweet sorghum syrup at 30 g/L total sugar and supplemented with 1.45 g/L $FeSO_4$. Throughout the experiment, the ASBR was operated at 24 hr HRT which is the optimum condition obtained from our previous study (Saraphirom and Reungsang, 2010c). A sequencing batch mode was operated at 12 hr of cycle period consisting of 20 min of filling period; 20 min of settling period; 20 min of decanting period, 11 hr of reacting period and medium replacement volume of 500 mL. The reactor was operated at room temperature (30±3 °C). The ASBR reached the steady state after 30 days with the volatile suspended solid (VSS) of 5.0 g/L. After the steady state was achieved, the ASBR was continuously operated to produce hydrogen from sweet sorghum syrup with 4 treatments (Table 2). Each treatment was operated in continuous manner. New treatment was introduced after the steady state of previous treatment was reached. The system was left in new condition for at least 10-15 times of HRT until the new steady state was reached in order to ensure there is no carryover of the

previous treatment. Constant substrate removal efficiency and hydrogen production (±5% variation) were considered as indicators for the steady state conditions. The gas produced was collected daily and the biogas volume was measured by water replacement method (Saraphirom and Reungsang, 2010b).

Table 2 Experimental set up

Treatment	Experimental set up
A	Nutrient + vitamin
B	Nutrient
C	No supplements (control)
D	Vitamin

7.2.5 Analytical methods

Biogas composition was measured by a gas chromatography (GC-2014, Shimadzu) equipped with a thermal conductivity detector (TCD) and 2 m stainless column packed with Shin carbon (50/80 mesh). The operational temperatures of the injection port, the column oven and the detector were 100, 120 and 150 °C, respectively. Helium was used as the carrier gas at a flow rate of 25 mL/min.

The liquid sample was first centrifuged at 10,000 rpm for 5 min, acidified by 0.2N oxalic acid and filtered through 0.2 μm nylon syringe filter before analyzed for volatile fatty acids (VFAs), acetone and alcohols concentration using a gas chromatography (GC-2014, Shimadzu) equipped with a flame ionization detector (FID) and a 30 m x 0.25 mm x 0.25 μm capillary column (Stabiwax). The temperatures of the injector and detector were 250 °C. The initial temperature of column oven was 50 °C for 2 min followed with a ramp of 15 °C/min for 12.6 min and to final temperature of 240 °C for 1 min. Helium was used as a carrier gas with a flow rate of 66 mL/min. Lactic acid was analyzed by HPLC (Shimadzu LC-10AD) with a UV detector (210 nm) and Prevail Organic Acid 5μ column (250 mm x 4.6 mm) using 25 mM KH_2PO_4 (pH 2.5) with a flow rate of 0.8 to 1.2 mL/min as the mobile phase.

The biomass (in term of volatile suspended solid (VSS)) and total sugar concentrations were determined by standard methods (APHA, 1995). The hydrogen

production rate (HPR) (L H_2/L-d) was calculated from hydrogen volume divided by fermentation time (1 day). Hydrogen volume is the sum of hydrogen volume at each time interval in one day. The HY was calculated as total molaric amount of hydrogen divided by molaric amount of consumed hexose (mol H_2/mol hexose consumed). The total molaric amount of hydrogen was calculated using the ideal gas law as: molar hydrogen production (mol H_2/L) = volumetric hydrogen production (L H_2/L)/(RT), where R = 0.08205784 L atm/K mol, and T = 303 K (Zhang et al., 2006).

7.2.6 Microbial community analysis

Sludge samples were collected from ASBR at steady state of Treatments A, B, C and D (Table 2). Total genomic DNA was extracted using the Ultraclean Soil DNA Kit (MoBio Laboratory Inc., USA). The region of the 16S rRNA genes corresponding to position 340–518 in the 16S rRNA of *Escherichia coli* was PCR-amplified using the forward primer; L340GCf (50-CCTACGGGAGGCAGCAG-30) with a GC clamp at the 50 end and the reverse primer; K517r (50-ATTACCGCGGCTGCTGG-30) (Muyzer et al., 1993). PCR amplification was conducted in an automated thermal cycler using the following protocol: initial denaturation for 5 min at 94 °C, 30 cycles of denaturation for 1 min at 95 °C, annealing for 30 s at 55 °C, extension for 1 min at 72 °C, followed by a final extension for 7 min at 72 °C. The DGGE analysis of the PCR products was performed by electrophoresis for 20 min at 20V and 15 hr at 70V through a 7.5% polyacrylamide gel containing a linear gradient of denaturant (100% denaturant corresponds to 7M urea and 40% (v/v) formamide deionized with AG501-X8 mixed bed resin) ranging from 30% to 60% in 0.5 x TAE buffer at a constant temperature of 60 °C (DGGE unit, V20-HCDC, Scie-Plas Limited, UK). The gel was stained with Sybr-Gold (1000x concentration) for 1 hr and visualized on a UV transilluminator. Most of the bands were excised from the gel and reamplified with the forward primer without a GC clamp and the reverse primer. After re-amplification, PCR products were purified using E.Z.N.A cycle pure kit (Omega Bio-tek, USA) and sequenced using primer K517r and an ABI PRISM Big Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, USA) in accordance with the manufacturer's instructions. Closest matches for partial 16S rRNA gene sequences were identified by database searches in GenBank using BLAST (Altschul et al., 1997).

7.3 Results and Discussion

7.3.1 Continuous hydrogen production

Figure 2 depicted the time course of continuous hydrogen production over 77 days in ASBR operated with OLR of 30 g total sugar/L-d at 24 hr HRT. The average range of ORP was 371 to 425 (-mV) which ensured the anaerobic condition. pH was controlled and stabled at 5.0±0.1 (Figure 2a).

Biogas and hydrogen production rates were rapidly increased after day 13 in the treatment with nutrient and vitamin solutions (Treatment A), then sharply decreased and stable at 7.2 L/L-d and 3.2 L H_2/L-d, respectively (Table 3) on day 18 to day 24 (Figure 2b). Supplemented with only nutrient solution (Treatment B), a gradually decrease in biogas and hydrogen production rates to 5.2 L/L-d and 2.0 L H_2/L-d, respectively, was found. The biogas and hydrogen production rates were significantly decreased (Table 3, Fig 2b) without the supplementations of nutrient and vitamin (Treatment C). However, the biogas and hydrogen production rates were improved with the supplementation of vitamin solution (Treatment D). These results indicated that nutrient and vitamin solutions have the profound effect on hydrogen production. The hydrogen content was varied at the beginning of each treatment and reached 40 to 44% at the steady state (Figure 2b) and the profiles of hydrogen content were similar to those of biogas and hydrogen production rates.

The maximum HY and HPR of 1.6 mol H_2/mol hexose and 3.2 L H_2/L-d (Table 3), respectively, were obtained when the fermentation broth was supplemented with nutrient and vitamin solutions (Treatment A) while a maximum specific hydrogen production rate (SHPR) of 0.83 L H_2/g MLVSS-d was obtained when only nutrient was added (Treatment B) (Figure 2c). Results indicated that nutrient and vitamin supplementations (Treatment A) improved the biogas as well as hydrogen production approximately 5 times in comparison to the control (Treatment C).

The fluctuations of biomass concentration, represented by VSS, were occurred at the start up of ASBR when nutrient and vitamin solutions (Treatment A) were added and then reached a constant of 5.3 g/L after day 12. VSS concentrations were decreased to 2.5 g/L when the treatment was switched to only nutrient supplementation (Treatment B) and reached a low concentration when no supplementations were added (1.7 g/L). Significant increased in VSS concentration

to 2.4 g/L was found when vitamin solution was added to the fermentation broth (Table 3, Figure 2c). These results indicated that nutrient and vitamin solutions are necessary for both hydrogen and biomass productions.

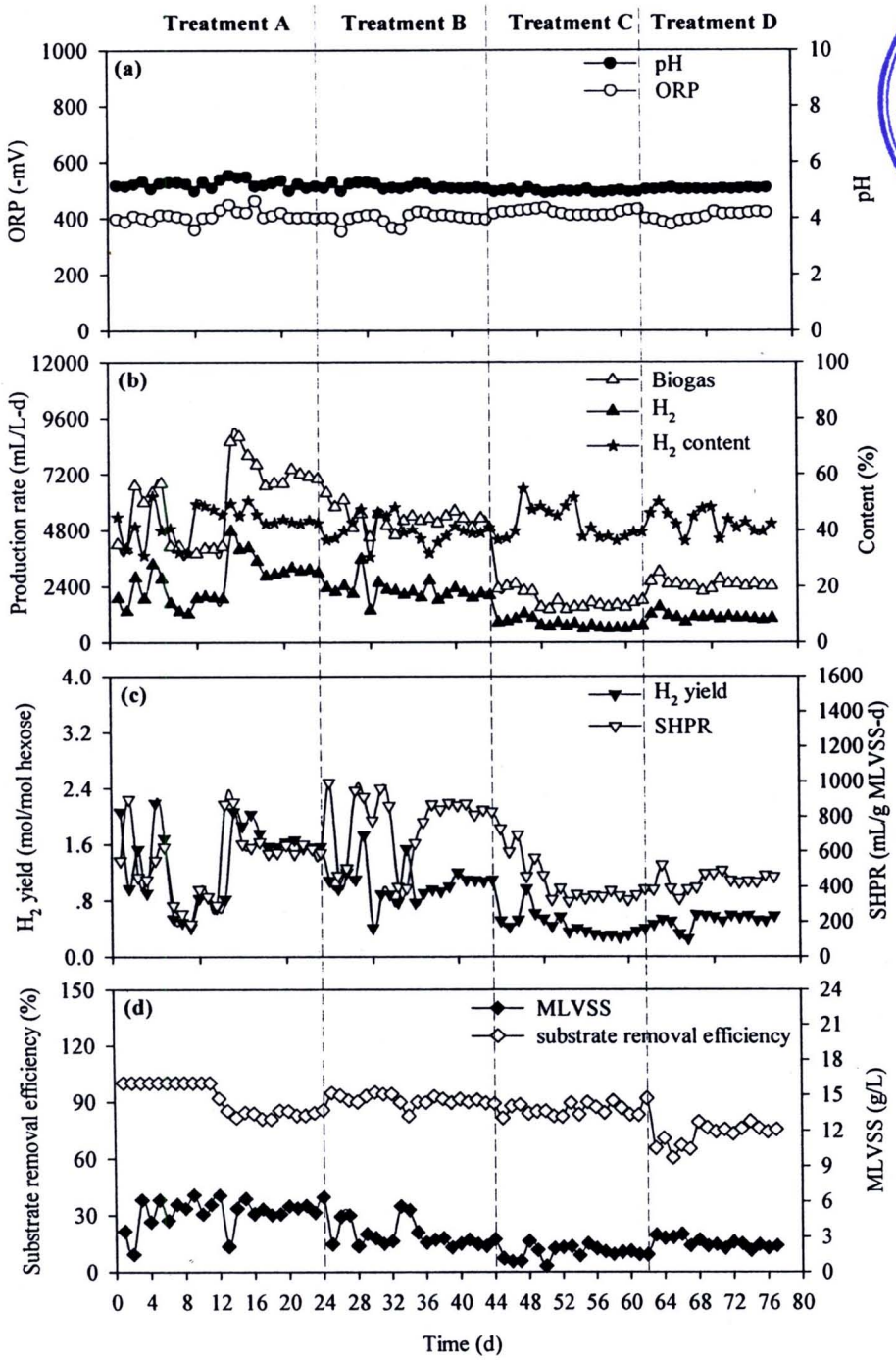

Figure 2 Profiles of hydrogen production performance a) pH and ORP, b) Bio gas production rate, HPR and hydrogen content c) HY and SHPR, d) MLVSS and substrate removal efficiency

A complete substrate consumed was observed at the beginning of ASBR operation until day 11 then slowly decreased and reached the steady state at the substrate removal efficiency of 80 to 83.5% in Treatment A (Figure 2d). Substrate removal efficiency slightly increased from 83.5% in Treatment A (Table 3) to 90.3% when only nutrient was added (Treatment B). Without the supplementations (Treatment C), a slightly decreased in the substrate removal efficiency (86.9%) was found. However, the supplement of only vitamin solution reduced the substrate consumed efficiency (73.3%).

Table 3 Summary of continuous biohydrogen production performance using different improvement methods.

Treatment / **Operational mode**	**A**	**B**	**C**	**D**
MLVSS (g/L)[a]	5.3±0.48	2.5±0.29	1.7±0.33	2.4±0.37
Substrate removal efficiency (%)[b]	83.5±1.81	90.3±1.04	86.9±3.34	73.3±5.62
Biogas production(L/L-d)	7.2±0.42	5.2±0.29	1.6±0.12	2.4±0.11
Hydrogen content (%)	44.0±2.65	40.1±1.91	40.6±5.18	42.4±3.74
HPR (L H_2/L-d)	3.2±0.03	2.0±0.15	0.6±0.07	1.0±0.07
HY (mol H_2/mol hexose)	1.6±0.20	1.0±0.10	0.34±0.04	0.52±0.11
SHPR (L H_2/g MLVSS-d)[c]	0.60±0.10	0.83±0.13	0.38±0.09	0.44±0.06

a Mixed liquor volatile suspended solid

b Substrate removal efficiency = ((Initial hexose-Residual hexose)/Initial hexose) x 100

c Specific hydrogen production rate

Nutrient solution (a modified Endo nutrient formula) supplemented to the sweet sorghum syrup to produce hydrogen by heat-treated anaerobic sludge in this study gave a significantly increase in hydrogen production. This is due to the fact that Modified Endo nutrient contains the important elements such as nitrogen, phosphate, magnesium, sodium, zinc and iron. Nitrogen and phosphorus are the important component for proteins, nucleic acids and enzymes that are of great significance to the growth of hydrogen producing bacteria (Wang and Wan, 2009). Phosphate, in the

form of K_2HPO_4 in a modified Endo nutrient formula, is needed for hydrogen production as well as buffering capacity. Iron is needed for the activity of hydrogenase which is an iron-containing enzyme directly responsible for hydrogen formation. It has been reported that the *in vivo* activity of hydrogenase in fermentative bacteria decreased with the depletion of iron (Dabrock et al., 1992). The essential of iron was also demonstrated for hydrogen production by *Clostridium*-rich composts (Lin and Lay, 2004), anaerobic mixed cultures (Zhang et al., 2005) and heat treated sludge (Saraphirom and Reungsang, 2010b). The other nutrients i.e. magnesium, sodium and zinc are necessary trace elements for the synthesis of various anaerobic microorganisms related to the bacterial enzyme cofactor, transport process and dehydrogenase (Lin and Lay, 2005).

Vitamins are necessary for the growth and metabolisms of microorganisms (Xu et al., 2008). For example, vitamin B_1 promotes the glycolysis pathway rate and vitamin B_2 accelerates the electron transfer which enhanced the metabolic flux of the glycolysis pathway (Sheng and Wang, 1991). In addition, Pan et al., (2008a) reported that the hydrogen production medium supplemented with vitamins solution consisted of vitamin B_{12}, vitamin C, riboflavin, citric acid, pyridoxal, folic acid and creatine which was supplemented to hydrogen production medium could improve the cell growth and the hydrogen production of *Clostridium* sp. Due to the aforementioned reasons, it can be concluded that the improvement of continuous hydrogen production from sweet sorghum syrup in ASBR might be due to the supportive relationship between the nutrient and vitamin.

The improvement of HY (from 0.34 to 1.6 mol H_2/mol hexose) by nutrient and vitamin supplementations obtained from this study was comparable to the CO_2 sparging method under mesophillic condition and more superior than N_2 sparging method (Table 4). Nutrient supplementations at a thermophilic condition could give more improvement on the HY (O-Thong et al., 2007) (Table 4). The high HY under thermophillic condition was due to the reduction solubility of hydrogen and alleviation inhibitory from hydrogen partial pressure (Hawkes et al., 2002; van Groerestijn et al., 2002). Optimization of HRT and OLR without nutrient and vitamin supplementations in order to improve HY was not satisfactory (Table 4). The HY was increased from 0.43 and 0.20 mol H_2/mol hexose to 0.68 and 0.53 mol H_2/mol

hexose when HRT and OLR were optimized, respectively, (Saraphirom and Reungsang, 2010b, c). Therefore, our results demonstrated that the improvement method using nutrient and vitamin supplementations were necessitate for a continuous hydrogen production from sweet sorghum syrup in ASBR.

Table 4 Comparison of continuous biohydrogen production using different improvement methods.

Improvement process	Substrate	Inoculums	Reactor	Operational condition	H_2 yield (mol H_2/mol hexose)		Reference
					Before	After	
Nutrient supplementation	POME	*Thermoanaero-bacterium* rich sludge	ASBR	Thermophillic (60 °C)	1.6	2.24	O-Thong et al., 2007
CO_2 sparging	Sucrose	Heat-treated sludge	CSTR	Mesophillic (35 °C)	0.77	1.68	Kim et al., 2006
N_2 sparging	Sucrose	Heat-treated sludge	CSTR	Mesophillic (35 °C)	0.77	0.95	Kim et al., 2006
Optimized the HRT	Sweet sorghum syrup	Heat-treated sludge	ASBR	Mesophillic (30-33 °C)	0.43	0.68	Saraphirom &Reungsang, 2010b
Optimized the OLR	Sweet sorghum syrup	Heat-treated sludge	ASBR	Mesophillic (30-33 °C)	0.20	0.53	Saraphirom &Reungsang, 2010c
Nutrient and vitamin supplementations	Sweet sorghum syrup	Heat-treated sludge	ASBR	Mesophillic (30-33 °C)	0.34 (control)	1.6 (Treatment A)	This study

7.3.2 Soluble metabolites production

Major VFAs detected in the fermentation broth were lactic acid, butyric acid, acetic acid and propionic acid while the major alcohol was ethanol (Table 5). Lactic acid was the main metabolite product with the concentration higher than 1.0 g/L in each treatment (Table 5). Lactic acid formation has been reported in some hydrogen producing cultures (Oh et al., 2004) under nutrient limitation condition (Dabrock et al., 1992) or inhibition by organic acid (Ko et al., 2004). High concentration of lactic acid in this present study might be caused by the present of lactic acid bacteria (LAB) i.e. *Enterococcus faecium* (Treatments B, C) and

Lactobacillus sp. (Treatments A, B, D) (see section 3.4) which produce lactic acid as a major metabolic end product of carbohydrate fermentation (Kim and Chun, 2005; Lee et al., 2006).

Table 5 Soluble metabolite production under different nutrient and vitamin supplementations.

Treatment	**A**	**B**	**C**	**D**
Soluble metabolite products				
Acetic acid (mg/L)	49.9±1.20	54.7±11.10	21.4±6.37	10.9±1.69
Propionic acid (mg/L)	11.7±2.22	6.6±1.51	2.2±1.70	1.1±1.10
Butyric acid (mg/L)	146.0±13.17	135.7±6.14	3.2±1.97	2.2±0.67
Lactic acid (mg/L)	1517.5±57.65	1388.9±21.47	1325.1±39.62	1024.3±95.83
Ethanol (mg/L)	0	0	1271.3±96.65	593.1±86.97
Total SMP[a]	1725.2±56.57	1585.9±40.95	2632.2±55.46	1631.7±91.28
B/A ratio[b]	2.9±0.27	2.5±0.44	0.14±0.04	0.20±0.05

a SMP = soluble metabolite products (acetic acid + propionic acid + butyric acid + lactic acid + ethanol)

b Butyric acid/Acetic acid ratio

A low HY obtained in the control treatment (Treatment C) and the treatment with vitamin supplementation (Treatment D) was correlated with the high concentration of lactic acid and alcohol (Table 5). Our results were consistent with the report by Levin et al. (2004) that the high concentration of lactic acid and alcohol found in dark fermentation is associated with low HY. In addition, bacteriocins, the proteins with bactericidal activity, excreted by LAB may inhibit the hydrogen producing bacteria including *Clostridium* (Noike et al., 2002). Bacteriocins are frequently found as the secondary metabolites produced by diverse microorganisms such as LAB and genus *Bacillus* (Godič and Bogovič, 2003). Although, the presence of lactic acid as a metabolite during hydrogen production is frequently regarded as a sign of lower hydrogen production, lactic acid has been reported for hydrogen production. For example, lactate in starch-containing medium was used by mixed

cultures to produce hydrogen with the formation of butyric acid as the major metabolite (Baghchehsaraee et al., 2009). Some *Desulfovibrio* species could use lactate as an electron donor for cell growth associated with the hydrogen production (Carepo et al., 2002).

Ethanol was not produced in the treatments supplemented with nutrients (Treatments A and B) whereas ethanol was the major alcohol produced in the treatments without the nutrient supplementation (Treatments C and D). Low mineral salts concentration might change the metabolic pathway from the hydrogen production to ethanol production (Dabrock et al., 1992). In addition, ethanol might be produced as the end product from degradation of sugar by LAB such as *Lactobacillus* sp. which could degrade 1 mol of glucose to 1 mol of lactic acid, 1 mol of ethanol, and 1 mol of carbon dioxide (Madigan et al., 2000).

A high hydrogen production (Table 3) was coincided with the absence of alcohol production in Treatments A and B (Table 5). It was speculated that the alcohol formation in metabolic pathway was blocked by some microorganisms in the mixed cultures. Mahyudin et al., (1997) reported that fermentative bacteria stain *Enterobacter aerogenase* could prevent the formation of alcohol by blocking the pathways from pyruvate to ethanol.

Butyric acid in the effluent of the treatments with nutrient supplementation (Treatments A and B) were significantly higher than treatments with no nutrient supplementation (Treatments C and D). Normally, the hydrogen producing bacteria i.e. *Clostridia* ferment glucose to butyric acid, acetic acid, CO_2 and H_2 as the end products through pyruvate in the glycolysis pathway (Das and Veziroglu, 2008). Results implied that the fermentation process of sugars, mainly glucose, could be improved by nutrient and vitamin supplementations. Results also indicated the correlation of hydrogen production (Table 3) to a significantly high butyric to acetic ratios (B/A ratio) obtained from Treatments A and B than Treatments C and D (Table 5). B/A ratio greater than 2.5 was found in Treatments A and B whereas B/A ratio of 0.1 to 0.2 was found in Treatments C and D (Table 3). Hussy et al. (2003) reported that B/A ratio are directly proportional to HY. Han et al. (2005) stated that a B/A ratio greater than 2.60 indicated an efficient hydrogen production by anaerobic microflora whereas a B/A ratio lower than 1 resulted in low efficiency of

biohydrogen production (O-Thong et al., 2009; Wang and Wan, 2008). Our results clearly indicated that nutrient and vitamin supplementations could improve the efficiency of hydrogen production from sweet sorghum syrup by anaerobic sludge in ASBR.

7.3.3 Microbial community

The sludge samples at steady state of 4 different treatments were collected and their corresponding bacterial communities were analyzed using the PCR-DGGE techniques. The results from the microbial community analysis suggested that the nutrient and vitamin supplementations affected the hydrogen production efficiency (HY and HPR). The difference of microbial community in each treatment was observed which suggested that there is no carryover effect from the previous treatment. Results indicated that microbial population was treatment-dependent with *Klebsiella* sp., *C. pasteurinum*, *E. faecium*, *Lactobacillus* sp., *Clostridium* sp., *C. roseum*, *C. beijerinckii*, *C. acetobytylicum* and *Desulfovibrio* sp. (Figure 3) are predominant species. Hydrogen producing bacteria i.e. *Clostridia* species were detected in every treatments while *Klebsiella* sp. and *Desulfovibrio* sp. were only found in treatments supplemented with nutrients (Treatment A and B). The higher hydrogen production obtained from the treatments with nutrient supplementation (Treatments A and B) might be due to the presence of Clostridia species together with *Klebsiella* sp. and *Desulfovibrio* sp. Clostridia species have been reported to be responsible for hydrogen production via dark fermentation (Lin et al., 2006). Hydrogen production by Clostridia species is accompanied with VFAs and/or solvent production (Lin et al., 2006). *Klebsiella* sp., a facultative anaerobe in family Enterobacteriaceae is known as solvent production strains (Wu et al., 2008). They are able to convert carbohydrates to a number of soluble and gaseous products such as 2,3 butanediol, ethanol, isopropanol, hydrogen and carbon dioxide (Wu et al., 2008). In addition, *Desulfovibrio* sp., has a capable to produce hydrogen when cultured under no limitation of sulfate and lactate (8.8 g/L sulfate or lactate), sulfate limitation (1-1.7 g/L sulfate), lactate limitation (1.1 g/L lactate) and in the presence of molybdate (20 mM) (Carepo et al., 2002).

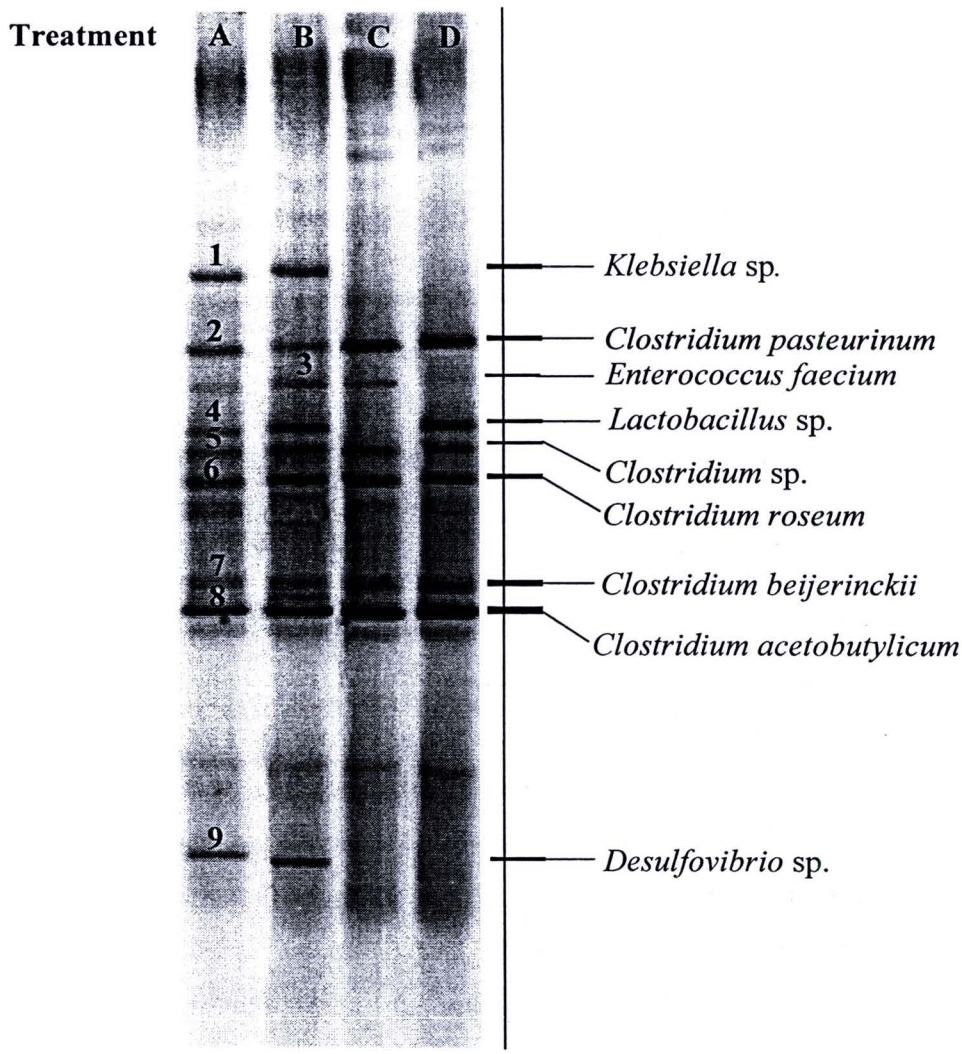

Figure 3 DGGE analysis of microbial community at steady state of 4 treatments, Lane 1: the treatment with nutrient and vitamin solutions (Treatment A); Lane 2: the treatment supplemented with only nutrient solution (Treatment B); Lane 3: the treatment without the supplementations of nutrient and vitamin (Treatment C) and Lane 4: the treatment supplemented with only vitamin solution (Treatment D)

Since the sulfate concentration in modified Endo nutrient and $FeSO_4$ used in this study was less than 1.7 g/L as well as the concentration of lactate produced in the fermentation broth was approximately 1-1.5 g/L (Table 5), thus the results implied that hydrogen was produced by *Desulfovibrio* sp. under sulfate or lactate limitations condition. Even though, the results indicated that nutrient addition

(Treatments A and B) could improve the hydrogen production, but the superior result was obtained in the treatment supplemented with nutrient and vitamin solutions (Treatment A). LAB i.e. *Enterococcus* sp. and *Lactobacillus* sp. were found in treatment supplemented with only nutrient solution (Treatment B) which coincided with a low HPR and HY obtained (Table 3). Noike et al., (2002) reported that LAB could inhibit hydrogen production by *Clostridia* species. Several strains of LAB such as Enterococci and Bacilli could produce bacteriocins, which have deleterious effect on hydrogen producing bacteria (Sarantinopoulos et al., 2002). Clostridia species were predominant microorganisms detected in the nutrient limitation treatments i.e. the control treatment (Treatment C) and the treatment with only vitamin added (Treatment D). Results indicated a low HPR and HY (Table 3) were obtained. Under nutrient limitation condition, Clostridia species produce other products such as alcohol as well as the production of hydrogen (Dabrock et al., 1992).

Results suggested that the lacking of nutrient supplementation was led to low hydrogen production ability of Clostridia species. In addition, the treatments supplemented with only vitamin solution (Treatment D) might be suitable for LAB i.e. *Enterococcus* sp. and *Lactobacillus* sp. which couased an adverse effect on hydrogen producing bacteria from the secreted bacteriocin.

7.4 Conclusions

Results indicated that nutrient and vitamin supplementations could increase HPR (3.2 L H_2/L-d) and HY (1.6 mol H_2/mol hexose) up to 5 folds in comparison to the control (0.6 L H_2/L-d and 0.34 mol H_2/mol hexose respectively). The improvement of continuous hydrogen production from sweet sorghum syrup in ASBR might be due to the supportive relationship between the nutrients and vitamins. A high B/A ratio of 2.5 to 2.9 in treatments with nutrient and vitamin supplementations indicated that the efficiency of hydrogen production was certainly improved. The PCR-DGGE analysis indicated that the predominant hydrogen producer were Clostridia species. The higher hydrogen production obtained from the treatments with nutrient supplementation (Treatments A and B) might be due to the presence of *Clostridia* species together with *Klebsiella* sp. and *Desulfovibrio*sp. Lack of nutrients in treatment without the supplementation (Treatments C) and in treatment with only

vitamin solution was added (Treatment D) could lead to the reduction of hydrogen production efficiency of *Clostridia* species. The presence of LAB i.e. *Enterococcus* sp. and *Lactobacillus* sp. caused an adverse effect on hydrogen producing bacteria resulted in a low HPR and HY in these two treatments.

7.5 References

[1] Kamalaskar LB, Dhakephalkar PK, Meher KK, Ranade DR. High biohydrogen yielding *Clostridium* sp. DMHC-10 isolated from sludge of distillery waste treatment plant. Int J Hydrogen Energy 2010;35:10639-44.

[2] Wilkie AC, Riedesel KJ, Owens JM. Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feed stocks. Biomass Bioenergy 2000;19:63-102.

[3] Tsai WT, Lin CC, Yeh CW. An analysis of biodiesel fuel from waste edible oil, in Taiwan. Renew Syst Energy Rev 2007;11:838-57.

[3] Xu GQ, Chu J, Zhuang YP, Wang YH, Zhang SL. Effects of vitamins on the lactic acid biosynthesis of *Lactobacillus paracasei* NERCB 0401. Biochem Eng 2008;38:189-97.

[4] Virje TD, Hass GGD, Tan GB, Keijsers ERP, Claassen PAM. Pretreatment of Miscanthus for hydrogen production by Themotoga elfi. Int J Hydrogen Energy 2007;27:1381-90.

[5] Ngo TA, Kim MS, Sim SJ. High-yield biohydrogen production from biodiesel manufacturing waste by *Thermotoga neapolitana*, Int J Hydrogen Energy (2010), doi:10.1016/j.ijhydene.2010.11.057

[6] Das D, Veziroglu TN. Hydrogen production by biological processes: a survey of literature. Int J Hydrogen Energy 2001;26:13–28.

[7] Liu Q, Zhang X, Zhou Y, Zhao A, Chen S, Qian G, Xu ZP. Optimization of fermentative biohydrogen production by response surface methodology using fresh leachate as nutrient supplement. Bioresource Technol (2011), doi: 10.1016/j.biortech.2011.03.002

[8] Li C, Fang HHP. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. Crit Rev Environ Sci Technol 2007;37:1-39.

[9] Lay CH, Wu JH, Hsiao CL, Chang JJ, Chen CC, Lin CY. Biohydrogen production from soluble condensed molasses fermentation using anaerobic fermentation. Int J Hydrogen Energy 2010;35:13445-51.

[10] O-Thong S, Prasertsan P, Intrasungkha N, Dhamwichukorn S, Birkeland NK. Improvement of biohydrogen production and treatment efficiency on plam oil mill effluent with nutrient supplementation at thermophilic condition using an anaerobic sequencing batch reactor. Enzyme Microb Technol 2007;41:583-90.

[11] Zhao C, O-Thong S, Karakashev D, Angelidaki I, Lu W, Wang H. High yield simultaneous hydrogen and ethanol production under extreme-thermophilic (70 °C) mixed culture environment. Int J Hydrogen Energy 2009;34:5657-65.

[12] Zhao BH, Yue ZB, Zhao QB, Mu Y, Yu HQ, Harada H, Li YY. Optimization of hydrogen production in a granule-based UASB reactor. Int J Hydrogen Energy 2008;33:2454-61.

[13] Saraphirom P, Reungsang A. Biological hydrogen production from sweet sorghum syrup by mixed cultures using an anaerobic sequencing batch reactor (ASBR). Int J Hydrogen Energy (2010b), doi:10.1016/j.ijhydene.2010.08.058

[14] Saraphirom P, Reunsang A. Effect of organic loading rate on biohydrogen production from sweet sorghum syrup by anaerobic mixed cultures in nnaerobic sequencing batch reactor. Int J Energy 2010c;4: 55-62.

[15] Muyzer G, DeWaal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S rRNA. Appl Environ Microbiol 1993;59:695–700.

[16] Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–402.

[17] Kim DH, Han SK, Kim SH, Shin HS. Effect of gas sparging on continuous fermentative hydrogen production. Int J Hydrogen Energy 2006;31:2158–69.

[18] Pattra S. Lay CH, Lin CY, O-Thong S, Reungsang A. Performance and population analysis of hydrogen production from sugarcane juice by non-sterile continuous stirred tank reactor augmented with *Clostridium butyricum*. Int J Hydrogen Energy (2010), doi:10.1016/j.ijhydene.2010.05.120

[19] Arooj MF, Han SK, Kim SH, Kim DH, Shin H-S. Continuous biohydrogen production in a CSTR using starch as a substrate. Int J Hydrogen Energy 2008;33:3289-3294.

[20] Jaisil P. Feasibility study on sweet sorghum production as raw materials for commercial ethanol production. In the Proceedings of the International conference on Agricultural, Food and Biological Engineering & Post Harvest/Production Technology.21-24 January 2007, Sofitel Raja Orchid Hotel, Khon Kaen, Thailand.

[21] Bulawayo B, Bvochora JM, Muzondo MI, Zvauya R. Ethanol production by fermentation of sweet-stem sorghum juice using various yeast strains World J Microbiol Biotechnol 1996;12:357-360.

[22] Laopaiboon L, Nuanpeng S, Srinophakun P, Klanrit P, Laopaiboon P. Ethanol production from sweet sorghum juice using very high gravity technology: Effects of carbon and nitrogen supplementations. Bioresource Technol 2009;100: 4176-82.

[23] Antonopoulou G, Hariklia N, Gavala, Ioannis V, Skiadas K, Angeloulos, Gerasimos L. Biofuels generation from sweet sorghum: Fermentative hydrogen production and anaerobic digestion of the remaining biomass. Bioresource Technol 2008;99:110-9.

[24] Saraphirom P, Reungsang A. Optimization of biohydrogen production from sweet sorghum syrup using statistical methods. Int J Hydrogen Energy 2010a; 35: 13435-44.

[25] Lin CY, Lay CH. A nutrient formulation for fermentative hydrogen production using anaerobic sewage sludge microflora. Int J Hydrogen Energy 2005;30:285-292.

[26] Su H, Cheng J, Zhou J, Song W, Cen K. Combination of dark- and photo-fermentation to enhance hydrogen production and energy conversion efficiency. Int J Hydrogen Energy 2009;34:8846-53.

[27] Wang J, Wan W. Factors influencing fermentative hydrogen production: A review. Int J Hydrogen Energy 2009;34:799-811.

[28] Bisaillon A, Turcot J, Hallenbeck PC. The effect of nutrient limitation on hydrogen production by batch cultures of *Escherichia coli*. Int. J. Hydrogen Energy 2006;31:1504-1508.

[29] Dabrock B, Bahl H, Gottschalk G. Parameters affecting solvent production by *Clostridium pasteurianum*. Appl Environ Microbiol 1992;58:1233-9.

[30] Lin CY, Lay CH. Carbon/nitrogen ratio effect on fermentative hydrogen production by mixed microflora. Int J Hydrogen Energy 2004;29:41–5.

[31] Zhang Y, Liu G, Shen J. Hydrogen production in batch culture of mixed bacteria with sucrose under different iron concentrations. Int J Hydrogen Energy 2005;30:855–60.

[32] Hawkes FR, Dinsdale R, Hawke DL, Hussy I. Sustainable fermentative hydrogen production: challenges for process optimization. Int J Hydrogen Energy 2002;27:1339-47.

[33] Sheng T, Wang JY. Biochemistry, 2nd ed., Higher Education Press, Beijing, 1991.

[34] van Groerestijn JW, Hazewinkel JHO, Nienoord M, Bussmann PJT. Energy aspects of biological hydrogen production in high rate bioreactors operated in the thermophilic temperature range. Int J Hydrogen Energy 2002;27:1141–7.

[35] Oh SE, Lyer P, Bruns MA, Logan BE. Biological hydrogen production using a membrane bioreactor. Biotechnol Bioeng 2004;87:119-27.

[36] Ko IB, Li YY, Satoh H, Noike T. Influence of HRT and sand substrate concentration on the continuous hydrogen production by anaerobic

microflora. In: Proceedings of 10th world congress on anaerobic digestion, vol. 4. p. 2242-45;2004.

[37] Kim M, Chun J. Bacterial community structure in Kimchi, a Korean fermented vegetable food, as revealed by 16S rRNA gene analysis. Int J Food Microbiol 2005;103:91–6.

[38] Lee JY, Kim CJ, Kunz B. Identification of lactic acid bacteria isolated from Kimchi and studies on their suitability for application as starter culture in the production of fermented sausages. Meat Sci 2006;72: 437–45.

[39] Levin DB, Pitt L, Love M. Biohydrogen production: prospects and limitations to practical application. Int J Hydrogen Energy 2004;29: 173–85.

[40] Noike T, Takabatake H, Mizuno O, Ohba M. Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. Int J Hydrogen Energy 2002;27:1367-71.

[41] Godič TK, Bogovič MB. Partial characterisation of bacteriocins produced by *Bacillus cereus* isolates from milk and milk products. Food Technol Biotechnol 2003;41:121-9.

[42] Mahyudin AR, Furutani Y, Nakashimada Y, Kakizono T, Nishio. Enhanced hydrogen production in altered mixed acid fermentation of glucose by *Enterobacter aerogenase*. J Ferm Bioeng 1997;83:354-63.

[43] Das D, Veziroglu TN. Advances in biological hydrogen production processes. Int J Hydrogen Energy 2008;33:6046–57.

[44] Hussy I, Hawkes FR, Dinsdale R, Hawkes DL. Continuous fermentative hydrogen production from a wheat starch co-product by mixed microflora. Biotechnol Bioeng 2003;84:619-36.

[45] Han SK, Kim SH, Shin HS. UASB treatment of wastewater with VFA and alcohol generated during hydrogen fermentation of food waste. Process Biochem 2005;40:2897-2905.

[46] O-Thong S, Prasertsan P, Birkeland N. Evaluation of methods for preparing hydrogen producing seed inocula under thermophilic condition

by process performance and microbial community analysis. Bioresource Technol 2009;100:909-18.

[47] Wang JL, Wan W. the effect of substrate concentration on biohydrogen production by using kinetic models. Sci China Ser B-Chem 2008;51:1110-7.

[48] Madigan MT, Martinko JM, Parker J. 2000. Brock Biology of Microorganisms. Prentice-Hall, Upper Saddle River, NJ.

[49] Chu CF, Ebie Y, Xu KQ, Li YY, Inamori Y. Characterization of microbial community in the two-stage process for hydrogen and methane production from food waste. Int J Hydrogen Energy 2010;35:8253-61.

[50] Chin HL, Chen ZS, Chou CP. Fedbatch operation using *Clostridiidium acetobutylicum* suspension culture as biocatalyst for enhancing hydrogen production. Biotechnol Prog 2003;19:383-8.

[51] Heyndrickx M, De Vos P, De Ley J. Fermentation characteristics of *Clostridium pasteurianum* LMG3285 grown on glucose and manitol. J Appl Bacteriol 1991;70:52-8.

[52] Hatchikian EC, Forget N, Bernadac A, Alazard D, Ollivier B. Involvement of a sigle periplasmic hydrogenase for both hydrogen uptake and production in some *Desulfovibrio* species. Res Microbiol 1995;146:129-41.

[53] Lin CY, Lee CY, Tseng IC, shiao IZ. Bio-hydrogen production from sucrose using base-enriched anaerobic mixed microflora. Process Biochem 2006;41:915-9.

[54] Wu KJ, Saratale GD, Lo YC, Chen WM. Simultaneous production of 2.3 butanediol, ethanol and hydrogen with a *Klebsiella* sp. strain isolated from sewage sludge. Bioresource Technol 2008;99:7966-70.

[55] Sarantinopoulos P, Leroy F, Leontopoulou E, Georgalaki MD, Kalantzopoulos G, Tsakalidou E, Vuyst LD. Bacteriocin production by *Enterococcus faecium* FAIR-E 198 in view of its application as adjunct starter in Greek Feta cheese making. Int J Food Microbiol 2002;72:125-36.

[56] Pan CM, Fan YT, Zhao P, Hou HW. Fermentative hydrogen production by the newly isolated *Clostrium beijerinchii* Fanp3. Int J Hydrogen Energy 2008b;33:5383-91.

[57] Pan CM, Fan YT, Xing Y, Hou HW, Zhang. Statistical optimization of process parameters on biohydrogen production from glucose by *Clostridium* sp. Fanp2. Bioresource Technol 2008a;99:3146-3154.

[58] Li SL, Kuo SC, Lin JS, Lee ZK, Wang YH, Cheng SS. Process performance evolution of intermittent-continuous stirred tank reactor for anaerobic hydrogen fermentation with kitchen waste. Int J Hydrogen Energy 2008;33:1522-31.

[59] Wang CH, Lin PJ, Chang JS. Fermentative conversion of sucrose and pineapple waste into hydrogen gas in phosphate-buffered culture seeded with municipal sewage sludge. Process Biochem 2006;41:1353-58.

[60] Baghchehsaraee B, Nakhla G, Karamanev, Margaritis A. Effect of extrinsic lactic acid on fermentative hydrogen production. Int J Hydrogen Energy 2009;34:2573-9.

[61] Carepoa M, Baptista JF, Pamplona A, Fauque G, Mouraa JJG, Reis MAM. Hydrogen metabolism in *Desulfovibrio desulfuricans* strain New Jersey (NCIMB 8313)—comparative study with *D. vulgaris* and *D. gigas* species. Anaerobe 2002;8:325–32.

[62] Zhang H, Bruns MA, Logan BE. Biological hydrogen production by *Clostridium acetobutylicum* in an unsaturated flow reactor. Water Res 2006;40:728-34.

[63] APHA. 1995. Stand Methods for the Examination of Water and Wastewater, 19th ed. American Public Health Association, Washington DC., USA.