CHAPTER VIII
POLYHYDROXYALKANOATES PRODUCTION FROM HYDROGENOGENIC EFFLUENT BY *Cupriavidus* sp. KKU38

8.1 Introduction

Polykydroxyalkanoates (PHAs) are a group of biologically derived biopolyester of hydroxyalkanoates that is accumulated as carbon/energy or reducing-power storage materials in microbial cells (Salehizadeh and Van Loosdrecht, 2004). When a carbon source is available in excess and other nutrients are growth-limiting, biopolyesters are deposited as water-insoluble cytoplasmic non-sized inclusions by *Eubacteria* and *Archaea* (Rehm, 2007).

Throughout the successful process of biohydrogen production, large amounts of organic wastewater were generated. This wastewater contained residual volatile fatty acids (VFAs) such as butyric and acetic acids and residual sugars, in case of sugary material is used to produce hydrogen, with high concentration of carbon oxygen demand (COD) which preventing the discharge of the effluent into the surrounding environment. VFAs had been used as valuable substrates for producing renewable energy such as ethanol and methane by various types of mixed and pure cultures (Ting et al., 2008; Luo et al., 2011) as well as producing PHAs by *Cupriavidus necator* (Hafuka et al, 2011). Compared to carbohydrates, lipids and amino acids, VFAs have simple structure with lower number of carbon atoms, which facilitates its easy synthesis to PHAs without the requirement of glycolysis and β-oxidation pathways by using less number of metabolic reactions and enzymes (Venkata Mohan et al., 2010).

PHAs production by mixed microbial cultures (Rodgers and Wu, 2010) is more energy-efficient than that involving pure culture (Kek et al., 2008; Lee et al., 2008). However, the PHAs content in the mixed cultures was not high (<65%) (Serafim

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et al., 2008) whereas high PHAs content (80%) was observed in pure culture (Li et al., 2007). In this study, *Cupriavidus* sp. KKU38 isolated from cassava starch manufacturing wastewater in our laboratory was used as the PHAs accumulating bacteria (Poomipuk et al., 2008). This strain could produce PHAs from sugars in cassava starch hydrolysate (Plangklang et al., 2010) and from VFAs in fermented cassava starch wastewater (Poomipuk et al., 2010). The preliminary results indicated that *Cupriavidus* sp. KKU38 was able to compete with the normal flora presented in the fermented cassava wastewater with the capability to accumulate PHAs of approximately 80% of dry cell weight (Poomipuk et al., 2010; Plangklang et al., 2010). Therefore, the possibility of employing *Cupriavidus* sp. KKU38 as the PHAs accumulator using VFAs and residual sugars in the effluent of hydrogen fermentation process as the substrate was investigated in this study.

The goal of this study was to explore to possibility of using the effluent discharged after hydrogen production from sweet sorghum syrup by anaerobic mixed cultures to produce PHAs by *Cupriavidus* sp. KKU38. The outcome from this study would not only add value to the wastewater by converting to valuable products i.e. PHAs but also to reduce its COD concentration before discharging into the environment.

8.2 Materials and Methods

8.2.1 Microorganism Preparation

8.2.1.1 Hydrogen producer

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.

8.2.1.2 PHAs producer

Cupriavidus sp. KKU38 is a PHAs accumulator isolated from cassava starch manufacturing wastewater. It was grown in nutrient broth (NB) in

250-ml flask at 30 °C and shaken at 150 rpm for 18 hr. The cells were then harvested by centrifugation at 10,000 rpm for 10 min and re-suspended in hydrogenogenic effluent before using as seed inoculum for PHAs production.

8.2.2 Hydrogenogenic effluent preparation

Hydrogen fermentation was conducted in anaerobic sequencing batch reactor (ASBR) with a total volume of 1.3 L (1 L liquid volume, 0.3 L gas holding capacity). 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 reactor. During the fermentation, 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.

The reactor 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 ASBR containing 900 mL of enrichment medium that consisted of sweet sorghum syrup at 30 g/L total sugar supplemented with 1.45 g/L $FeSO_4$. After 24 hr of reactor operation, 500 mL of the fermentation broth was replaced by the fresh enrichment medium and the reactor was operated again for 24 hr. The medium replacement was repeated 5 times in order to obtain a stable microbial community before starting the sequencing batch hydrogen fermentation. The hydrogen production medium contained 30 g/L total sugar of sweet sorghum syrup supplemented with 1.45 g/L $FeSO_4$, 10 mL/L of modified Endo nutrient solution and 10 mL/L of vitamin solution (Table 1). The hydrogen production medium was fed to the reactor with 24 hr hydraulic retention time (HRT). The ASBR was purged with nitrogen gas for 15 min to create anaerobic condition before operating in a sequencing batch mode operation at 12 hr of cycle period consisting of 20 min of filling period; 20 min of settling period; 20 min of decanting period and 11 hr of reacting period. The volume of filling and decanting step in the ASBR operation were 500 mL. The gas produced was collected daily and the biogas volume was measured by water replacement

method (Saraphirom and Reungsang, 2010b). The conditions used for hydrogen fermentation in ASBR were the optimum conditions obtained from the previous studies (Saraphirom and Reungsang, 2010a, b, c). After the constant substrate consumption, hydrogen production rate (HPR) and hydrogen yield (HY) (±5% variation) were achieved, the hydrogenogenic effluent from ASBR was collected and centrifuged at 5,000 rpm for 10 min. The pellets were removed and the resulted effluent was then used as the substrate for PHAs production.

Table 1 Compositions of modified Endo nutrient (modified from Lin and Lay, 2005) and vitamin solution (modified from Su et al., 2009).

Composition	Concentration (g/L)
Modified Endo nutrient	
NH_4HCO_3	524
K_2HPO_4	125
$MgCl_2.6H_2O$	100
$MnSO_4.6H_2O$	15
$CuSO_4.5H_2O$	5
$CoCl_2.5H_2O$	0.125
$NaHCO_3$	672
Vitamin solution	
Glutamic acid	0.01
Ascorbic acid	0.025
Riboflavin	0.025
Citric acid monohydrate	0.02
p-aminobenzoic acid	0.01
Creatinine	0.025

8.2.3 PHAs production

The batch-PHAs production from hydrogenogenic effluent by *Cupriavidus* sp. KKU38 was conducted in 250 mL Erlenmeyer flask, 100 mL working volume, containing 70 mL of hydrogenogenic effluent (pH 7.0) and 30 mL of seed inoculum (inoculum size of 30% (v/v), initial cell concentration of 10^7 CFU/ml). Flasks were incubated at 30 °C and shaken at 150 rpm for 12 days. Culture

medium was taken at the interval time for 288 hr to determine for the biomass, PHAs, total sugar, VFAs and COD concentrations. The PHAs production from hydrogenogenic effluent without inoculation of KKU38 was parallel conducted as the control.

8.2.4 Analytical methods

8.2.4.1 Biogas analysis

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.

8.2.4.2 Liquid sample analysis

The hydrogenogenic effluent and PHAs production broth were 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 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 high performance liquid chromatography (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.

Cell growth was evaluated by measuring the cell dry weight by using the standard method (APHA, 1995). PHAs concentration was analyzed employing the methods described by Usman-Arshad et al. (2007). Total sugar, total nitrogen, total phosphorus and COD concentrations were determined by standard methods (APHA, 1995). The 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).

8.3 Results and discussion

8.3.1 Hydrogen production

Hydrogen fermentation from sweet sorghum syrup supplemented with modified Endo nutrients and vitamins solution were conducted in ASBR to obtain the hydrogenogenic effluent as substrate for PHAs production. At steady state, biogas production rate was approximately 7.2 L/d with 44% hydrogen content. The maximum HPR of 3.2 L H_2/L-d and HY of 1.6 mol H_2/mol hexose were obtained. The compositions of the clarified hydrogenogenic effluent obtained are presented in Table 2. The COD concentration in the effluent was 26.7 g/L. Residual sugar was the highest fraction (8.78 g/L) detected. Lactic acid was the major VFAs product along with relatively low butyric, acetic and propionic acids, respectively. These soluble organic compounds have been reported as the substrate for PHAs production in numerous reports (Tong and Chen, 2007; Meungmeng et al., 2009; Yuan et al., 2006; Chen et al., 2007; Yan et al., 2003). The ratio of COD:N:P of the effluent was 100:0.53:0.02 indicated that the effluent is a carbon excess with limited nitrogen and phosphorus condition which is suitable for PHAs production (Majone et al., 1999; Serafim et al., 2004). Therefore, the soluble metabolites data showed the potential of hydrogenogenic effluent as the substrate for PHAs production.

8.3.2 PHAs production

8.3.2.1 Substrate utilization

The profiles of substrate utilization during PHAs production are presented in Figure 1. Soluble organic compounds i.e., residual sugar (hexose), and VFAs (lactic, acetic, butyric and propionic acids) in the hydrogenogenic effluents are considered as substrates in this study. Residual sugar was rapidly consumed in both treatments i.e. with and without the inoculum. The concentration of residual sugar decreased from approximately 8 g/L to 2.7 g/L within 36 hr and tended to be stable at

the concentration of less than 2.7 g/L until the end of incubation in the treatment with *Cupriavidus* sp. KKU38 (Figure 1a). During residual sugar was consumed, acetic acid concentration was found to increase continuously and then decreased when the residual sugar concentration was stable (Figure 1b). The results implied that *Cupriavidus* sp. KKU38 and/or indigenous microorganisms in the hydrogenogenic effluent converted sugar to acetic acid during cultivation. Acetic acid was almost completely depleted at 216 hr of incubation in both treatments (Figure 1b). The decrease in propionic acid concentration started when acetic acid was almost depleted which suggested that acetic acid is more favorable for the microorganisms than propionic acid (Figure 1c).

Table 2 Compositions of hydrogenogenic effluent

Parameter	Value
pH	4.9 ±0.03
COD (g/L)	26.7 ±1.90
Total sugar (g/L)	8.78 ±2.10
Total volatile fatty acid (g/L)	2.65 ±0.47
Acetic acid (g/L)	0.50 ±0.01
Propionic acid (g/L)	0.12 ±0.01
Lactic acid (g/L)	1.52 ±0.02
Butyric acid (g/L)	0.86 ±0.03
Total Kjeldahl nitrogen (g/L)	0.67 ±0.03
Total phosphorus (g/L)	0.02 ±0.002
COD:N:P ratio	100:0.53:0.02

Butyric acid was simultaneously consumed with residual sugar in which the concentration of butyric acid decreased from approximately 75 to 42 mg/L after 36 hr of incubation in the treatment with *Cupriavidus* sp. KKU38 (Figure 1d). In control treatment, the butyric concentration was gradually decreased then reaches to stable at approximately of 35.5 mg/L after 60 hr until the end of incubation. In the treatment with *Cupriavidus* sp. KKU38, the consumption of butyric acid was

stable at 36 hr when the peak of acetic concentration was observed and the consumption started again after 168 hr when the acetic acid was almost completely consumed (Figure 2b). These might be because acetic acid is less toxic and easier to be consumed by microorganisms than butyric acid (Van Ginkel and Logan, 2005). Thus, after acetic acid is produced and accumulated in the system, the microorganisms prefer to consume acetic without the consumption of butyric acids.

The profiles of lactic acid consumption are presented in Figure 1e. The results indicated that lactic acid consumption was very slow from 0 to 192 hr of incubation. The concentration of lactic acid decreased rapidly after 192 hr indicating that lactic acid was consumed and *Cupriavidus* sp. KKU38 could adapt themselves to utilize lactic acid when other VFAs were exhausted.

8.3.2.2 TVFAs profile and pH variation

A continuous decrease in TVFAs concentration was observed during PHAs production in which the higher TVFAs removal was observed in the treatment with *Cupriavidus* sp. KKU38 (Figure 2a). The pH of the system was relatively stable from 0 to 72 hr of incubation time during the reducing sugar was converted to acetic acid and butyric acid was consumed (Figure 2b). After 72 hr, pH was found to increase from approximately 8.4 to 9.6 which coincided with a decrease in TVFAs concentration, suggesting that VFAs in the hydrogenogenic effluent was consumed by *Cupriavidus* sp. KKU38.

8.3.2.3 Biomass and PHAs production

Batch cultivation of *Cupriavidus* sp. KKU38 using hydrogenogenic effluent as substrate was conducted. Hydrogenogenic effluent without inoculation was used as control. In the treatment with *Cupriavidus* sp. KKU38, cell biomass was gradually developed and reached the maximum value of 2.15 g/L at 288 hr of incubation (Figure 3a). The concentration of PHAs gradually increased to the maximum value of 0.85 g/L at 96 hr of incubation and slightly decreased thereafter (Figure 3b). The results implied that the utilizable substrates for *Cupriavidus* sp. KKU38 in the effluent might be exhausted after 96 hr of incubation, thus *Cupriavidus* sp. KKU38 consumed the storage PHAs for their survival and growth resulting in a decrease in PHAs concentration. Reis et al. (2003), Salehizadeh and Van Loosdrecht (2004) also reported the similar trend. In their study, the mixed

cultures were used to produce PHAs and they found that during the excess of external carbon substrate, the microbial uptake is driven to simultaneous growth and polymer storage. After substrate exhaustion, the stored polymer can be used as energy and carbon sources.

The biomass concentration in the control treatment was stable at approximately 0.70 g/L until 192 hr of incubation (Figure 3a). An increase in biomass concentration in the control treatment was observed at 216 hr (Figure 3a) which coincided with the rapid decrease in VFAs concentration especially lactic acid (Figure 1). The results indicated the success adaptation of indigenous microorganisms in the effluent to utilize the soluble organic compounds in the hydrogenogenic effluent for biomass production. The PHAs concentration in control treatment was considered low at approximately 0.07 g/L compared to 0.85 g/L in the inoculated treatment (Figure 3b) throughout the experiment which suggested that PHAs observed in the inoculated treatment was produced mainly by *Cupriavidus* sp. KKU38.

The content of PHAs in the cells in control treatment was low and varied between 0 and 17% (Figure 3c) while PHAs content in the treatment with *Cupriavidus* sp. KKU38 increased from 25.00 to 71.40% after 72 hr of incubation and decreased rapidly towards the end of 288 hr of incubation (Figure 3c). This might be due to the fact that the carbon sources i.e. residual sugar and acetic acid were utilized to produce the cells and PHAs at the early stage (0-72 hr) of cultivation and then after 72 hr, the PHAs production might be shifted to produce the cells biomass instead. Figure 3 indicated the reduction of PHAs content in the cells since the production of PHAs was ended while the cell biomass production of *Cupriavidus* sp. KKU38 was still continued. The decrease in PHAs content also implied that the stored PHAs of *Cupriavidus* sp. KKU38 were consumed since the utilizable substrates were exhausted. In addition, the results also indicated that the microorganisms tend to use substrates for cell biomass production without the accumulation of PHAs after 72 hr of incubation. It is well known that the efficiency of cell biomass and PHAs production were affected by the ratio of carbon to nitrogen (C/N) and/or carbon to phosphorus (C/P). Under suitable ratio (approximately 100:10:1), the substrate would be used for cell biomass production without an accumulation of PHAs. In contrast,

under the excess of external carbon source with the limitation of N or P, the storage of PHAs would occur (Rehm, 2007).

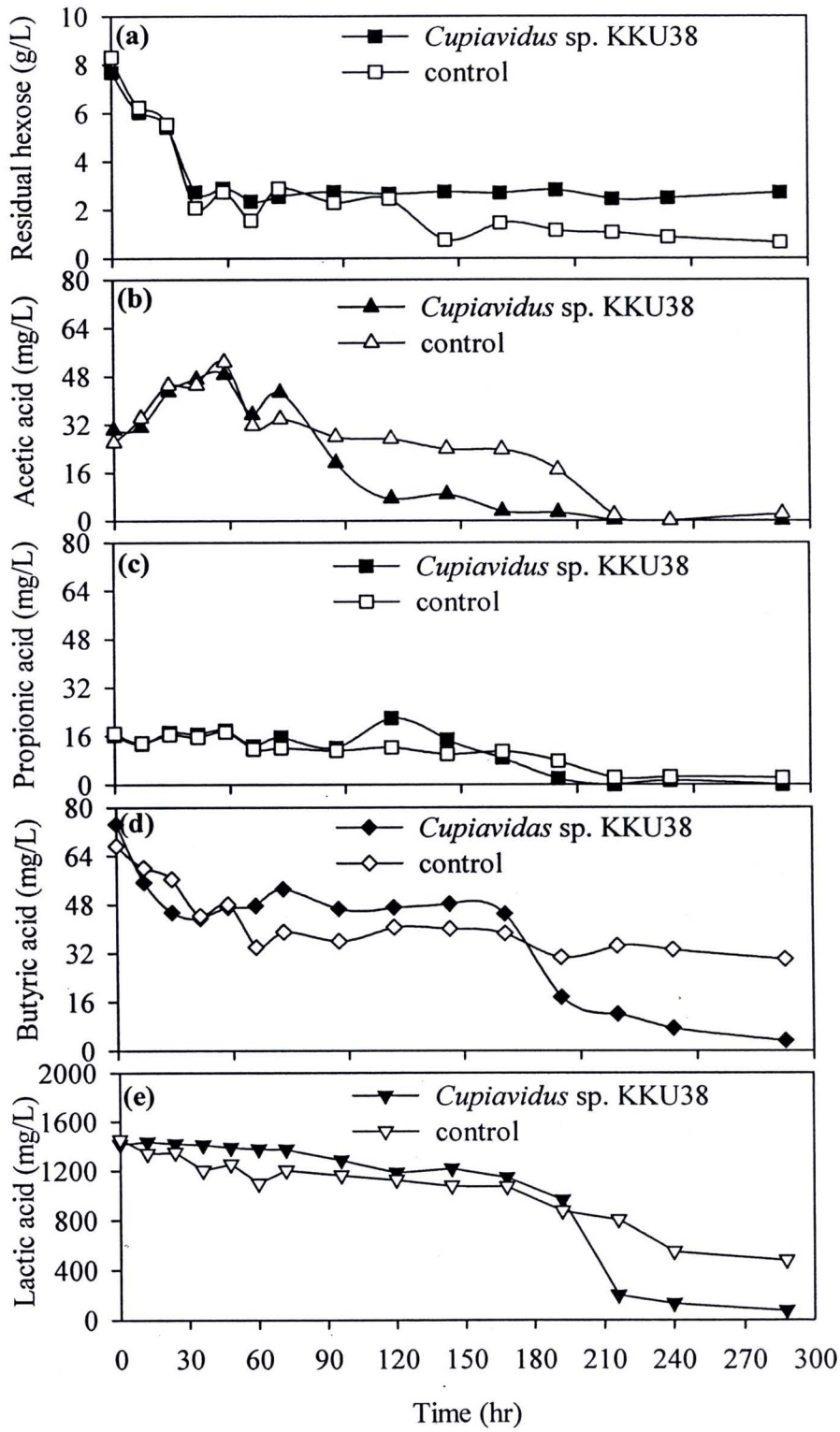

Figure 1 Substrate utilization profile.

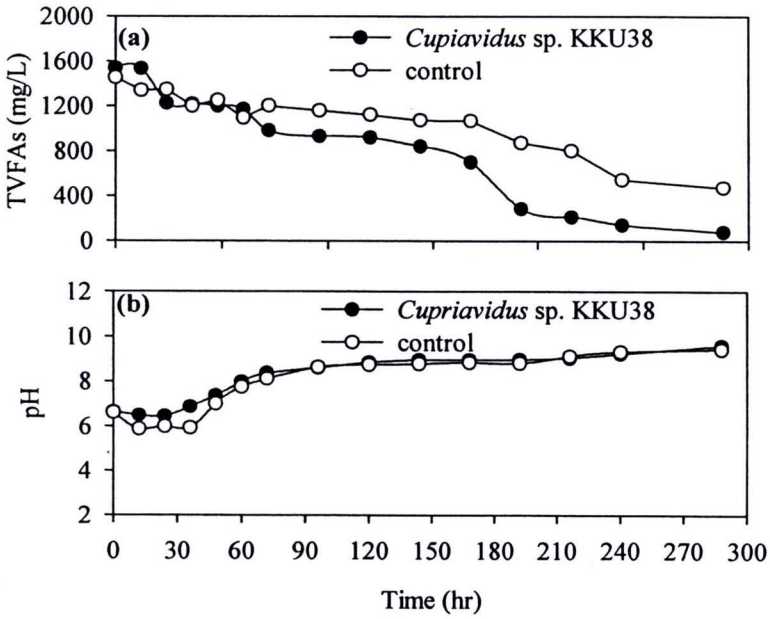

Figure 2 Total volatile fatty acid and pH profile.

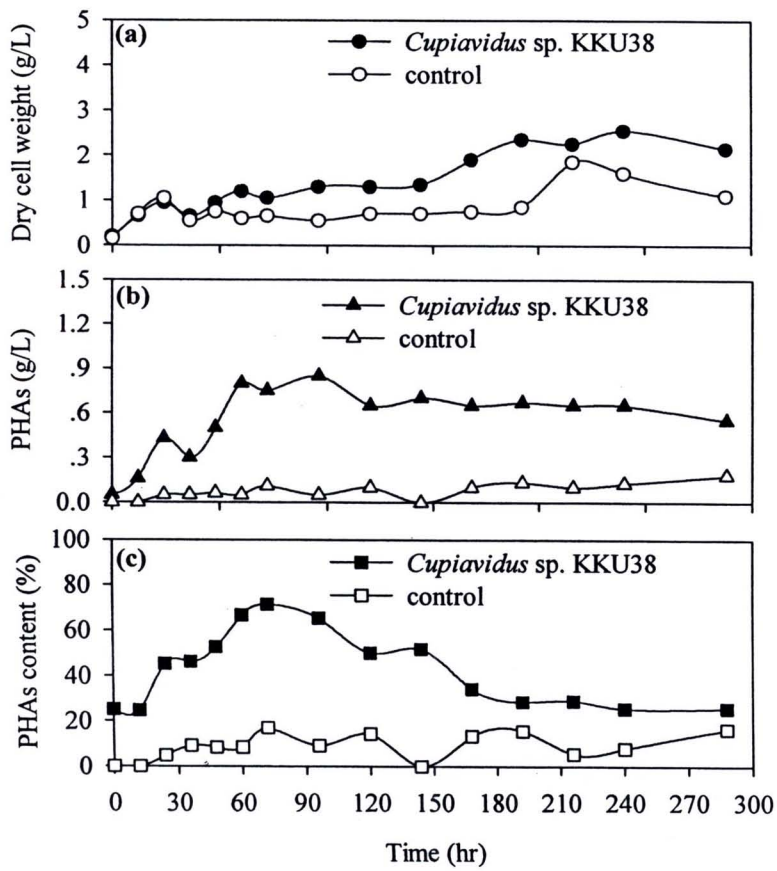

Figure 3 Dry cell weight of *Cupriavidus* sp. KKU38, PHAs concentration and PHAs content profiles.

8.2.3.4 COD profile

COD removal efficiency was investigated during PHAs production from hydrogenogenic effluent. The COD concentration profiles are presented in Figure 4. The concentration of COD decreased continuously during 288 day of incubation. The higher rate of COD removal was observed in the treatment with *Cupriavidus* sp. KKU38. Throughout the experiment, the COD was effectively removed with the removal percentage of approximately 82 and 68% for treatment with *Cupriavidus* sp. KKU38 and control, respectively. The results indicated that *Cupriavidus* sp. KKU38 efficiently utilized the soluble organic metabolites in the hydrogenogenic effluent for the growth and/or PHAs production.

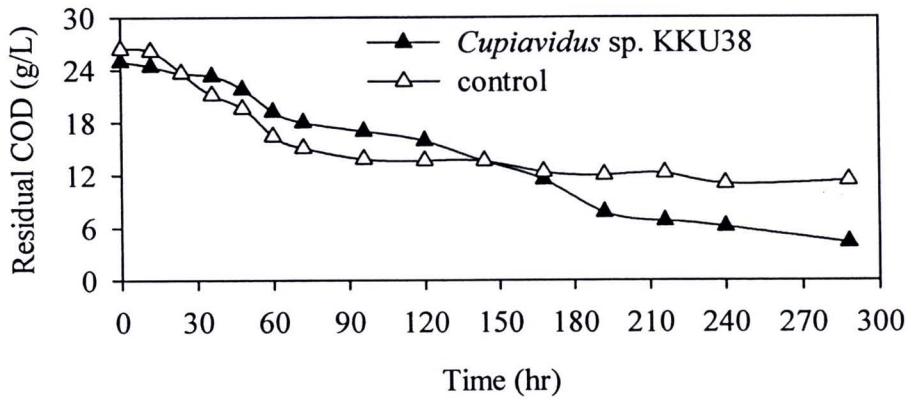

Figure 4 COD profile.

8.2.3.5 Performance of PHA production as compared to the other studies

Table 3 tabulated the performance of PHAs production in the treatment with *Cupriavidus* sp. KKU38 and in control at 72 hr which was the incubation time that the maximum PHAs content was obtained. *Cupriavidus* sp. KKU38 produced PHAs with a maximum PHAs content of 71% which suggested that the organic compositions in the hydrogenogenic effluent were suitable for PHAs production. The productivity and yields of biomass and PHAs were relatively low which suggested that the optimization of the PHAs production processes is needed. The examples of possible strategies for improving the productivity and the yields of biomass and PHAs are feeding adjustment and optimization of oxygen and initial inoculum concentrations in the system (Goff et al., 2007).

Table 3 Performance of PHAs production in treatment with *Cupriavidus* sp. KKU38 and control at 72 hr

Treatment	Biomass			PHAs			
	Conc. (g/L)	Productivity (g/L-hr)	Yield (g/g-COD consumed)	Conc. (g/L)	Productivity (g/L-hr)	Yield (g/g-COD consumed)	Content (% w/w)
-With *Cupriavidus* sp. KKU38	1.05	0.014	0.122	0.75	0.01	0.10	71.42
-Control (without inoculation)	0.65	0.009	0.044	0.11	0.002	0.01	16.92

The performance of PHAs production from hydrogenogenic effluent by *Cupriavidus* sp. KKU38 in comparison to the other literature search in term of PHAs content and PHAs yield is presented in Table 4. The PHAs content was comparable to the other studies which used hydrogenogenic effluent as substrate for PHAs production. However, the PHAs yield of 0.10 g PHAs/g-COD consumed obtained in this study was relatively low.

Table 4 Performance of PHAs production from hydrogenogenic effluent by *Cupriavidus* sp. KKU38 in comparison to the other literature search.

Substrate for hydrogen production	PHAs seed inoculum	Max. PHAs content (%)	Max. PHAs yield (g-PHAs/g-COD consumed)	Reference
Paper mill wastewater	Enriched activated sludge	48	0.11	Bengtsson et al., 2008
Palm oil mill wastewater	*Comamonas* sp. EB172	59	Nd	Zakaria et al., 2010
Olive oil mill wastewater	Enriched mixed	9	< 0.096	Ntaikou et al., 2009
Taihu blue algae	*Pseudomonas* sp. *Bacillus cereus*	43	nd	Yan et al., 2010
Molasses	Anaerobic sludge from hydrogen fermentation reactor	56	nd	Albuqerque et al., 2001
Sweet sorghum syrup	*Cupriavidus* sp. KKU38	71	0.10	This study

8.4 Conclusion

The possibility of PHAs production and COD removal from hydrogenogenic effluent of sweet sorghum syrup fermented stream by *Cupriavidus* sp. KKU38 was investigated. Results indicated that *Cupriavidus* sp. KKU38 effectively utilized soluble organic compounds in the effluent to produce PHAs with a maximum PHAs content of 71% and PHAs yield of 0.10 g/g COD consumed. Residual sugar and butyric acid in the hydrogenogenic effluent were the major substrates used to produce PHAs by *Cupriavidus* sp. KKU38. In addition, COD in the effluent was removed up to 80%.

8.5 Reference

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