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Optimum Conditions for the Production of Polyhydroxybutyrate from Cassava Wastewater by the Newly Isolated *Cupriavidus* sp. KKU38

(Keadaan Optimum untuk Penghasilan Polihidroksibutrat daripada Air Buangan Cassava menggunakan Isolat Baru *Cupriavidus* sp. KKU38)

S. SANGYOKA*, N. POOMIPUK & A. REUNGSANG

ABSTRACT

The Cassava starch manufacturing wastewater (CSW) was used as a substrate to produce polyhydroxybutyrate (PHB) by Cupriavidus *sp. KKU38. The acidogenic fermentation process of CSW was first conducted to obtain volatile fatty acids (VFAs), which are more efficient in PHB production than raw CSW. The effect on substrate concentration and nutrients, i.e. nitrogen and phosphorus concentrations, by means of chemical oxygen demand: nitrogen: phosphorus ratio (COD:N:P ratio) variation was investigated. The results indicated that PHB production from fermented CSW by* Cupriavidus *sp. KKU38 was optimized at the soluble COD:N:P ratio of 100:0.5:11. This ratio gave the maximum PHB content and yield of 85.53% and 0.31 g PHB/g COD consumed, respectively. By using the proposed PHB production process, the potential to produce 0.19 kg of PHB from 1.0 kg of soluble chemical oxygen demand (sCOD) contained in CSW was exhibited. The relatively high COD removal efficiency of 73.82% at the optimal condition could be achieved, which demonstrated the concept of water quality improvements alongside the production of the value-added by-product, PHB.*

Keywords: Cassava wastewater; Cupriavidus *sp. KKU38; polyhydroxybutyrate (PHB)*

ABSTRAK

Air buangan daripada penghasilan kanji Cassava (CSW) telah digunakan sebagai substrat untuk menghasilkan polihidroksibutrat (PHB) menggunakan Cupriavidus *sp. KKU38. Proses fermentasi asidogenik CSW dijalankan pada peringkat awal untuk mendapatkan asid lemak meruap (VFAs) yang lebih efisien dalam penghasilan PHB berbanding CSW mentah. Kesan kepekatan substrat dan nutrien iaitu kepekatan nitrogen dan fosforus menggunakan permintaan kimia oksigen: nitrogen: nisbah fosforus (nisbah COD:N:P) telah dikaji. Hasil kajian menunjukkan bahawa penghasilan PHB daripada fermentasi CSW melalui* Cupriavidus *sp. KKU38 telah dioptimumkan pada nisbah COD:N:P, 100:0.5:11. Nisbah ini memberikan masing-masing kandungan dan hasilan PHB 85.53% dan 0.31 g PHB/g COD yang digunakan. Dengan menggunakan proses penghasilan PBH ini, potensi untuk menghasilkan 0.19 kg PHB daripada 1.0 kg permintaan oksigen kimia terlarut (sCOD) yang terkandung dalam CSW telah ditunjukkan. Kecekapan pembuangan COD yang tinggi iaitu 73.82% pada keadaan optimum telah dapat dicapai. Ini menunjukkan bahawa konsep peningkatan kualiti air bersama penghasilan PHB dengan nilai tambah telah dapat dicapai.*

Kata kunci: Air buangan Cassava; Cupriavidus *sp. KKU38; polihidroksibutrat (PHB)*

INTRODUCTION

Plastics have become an integral part of contemporary life due to their versatile qualities of lightness, durability and resistance to degradation. These non-degradable plastics accumulate in the environment at a rate of millions of tons per year and cause several problems. Recent issues concerning the global environment and solid waste management have created a lot of interest in the development of biodegradable plastics (Anderson & Dawes 1990). Poly-β-hydroxyl butyrate (PHB) is the best characterized of all of the polyhydroxyalkanoates (PHAs) storage materials found in many bacteria (Madison & Huisman 1999). PHB has recently attracted much commercial interest as a plastic material because its physical properties are remarkably similar to polypropylene and polyethylene. It is readily biodegradable in both aerobic and anaerobic conditions. However, the current cost of the PHB production is considerably more than that of the synthetic plastics. Therefore, techniques for cost reduction such as strain development, cultivation and downstream process strategy improvement are required along with the search for inexpensive substrates for PHB production (Choi & Lee 1999).

In this study, the possibility of using CSW as a native-low cost carbon source for PHB production was investigated. *Cupriavidus* sp. KKU38, a newly isolated PHB producer from CSW was used as a seed inoculum. The effects of substrate concentrations and COD:N:P ratios in culture medium were examined in order to obtain suitable conditions for PHB and biomass production as well as COD reduction when the CSW was used as a substrate.

MATERIALS AND METHODS

CASSAVA WASTEWATER

Cassava starch manufacturing wastewater (CSW) was obtained from National Starch and Chemical Ltd., Kalasin Province, Thailand. The suspended solids in CSW were removed by simple gravity settling and the clear phase of CSW was used as a substrate for PHB production. Due to the previous research evidenced that volatile fatty acids (VFAs) were the efficient substrates to produce PHB (Yu 2001), the CSW was fermented to obtain VFAs before used to produce PHB in comparison to non-fermented CSW. The fermentation process was conducted in a 5 L laboratory glass bottle using anaerobic sludge from UASB wastewater treatment plant of beer industry (Khon Kaen Brewery Co. Ltd., Thailand) as a seed inoculum. The bottle was shaken at 150 rpm at room temperature for 48 h. The biogas produced was released via the port arranged at the top of the bottle. The suspended solids were removed and the clear phase of fermented CSW (FCSW) was used as a substrate for PHB production. The chemical characteristics of CSW and FCSW were shown in Table 1.

MICROORGANISM PREPARATION

Cupriavidus sp. KKU38 is a newly PHB producer isolated from cassava starch manufacturing wastewater and used to produce PHB in this study. It was grown in nutrient broth in 250 mL with incubated at 30ºC, shaken at 150 rpm for 18 h. The culture medium was then harvested by centrifugation at 10,000 rpm for 10 min and resuspended in 0.85% NaCl before using as seed inoculum for PHB production.

CULTIVATION MEDIUM

The E2 medium used for preparation of starter culture consists of 3.5 g of $NaNH_4HPO_4{\cdot}4H_2O$, 7.5 g of $K_2HPO_4{\cdot}3H_2O$, 3.7 g of KH_2PO_4 per liter supplemented with 10 mL of 100 mM $MgSO_4{\cdot}7H_2O$ and 1 mL microelements stock solution. One liter of microelements stock solution containing 2.78 mg of $FeSO_4{\cdot}7H_2O$, 1.98 mg of $MnCl_4{\cdot}4H_2O$, 2.81 mg of $CoSO_4{\cdot}7H_2O$, 1.47 mg of $CaCl_2{\cdot}2H_2O$, 0.17 mg of $CuCl_2{\cdot}2H_2O$ and 0.29 mg of $ZnSO_4{\cdot}7H_2O$ (Lageveen et al. 1988). The initial pH of E2 medium was 7. NH_4Cl and KH_2PO_4 were used to adjust the N and P concentrations in culture medium to obtain the expected COD:N:P ratios values in the experiment.

PHB PRODUCTION USING CSW OR FCSW AS SUBSTRATE

The batch-PHB production from CSW or FCSW by *Cupriavidus* sp. KKU38 was conducted in a 250 mL Erlenmeyer flask with the working volume of 100 mL. The effects of soluble COD (sCOD) concentration in the substrate on PHB production sCOD removal were firstly investigated. E2 medium contained with the various CSW or FCSW concentrations of 20, 40, 80 and 100% (v/v) with the fixed ratio of sCOD:N:P of 100:1:11 were used with the inoculum size of 30% (v/v) (initial cell concentration of 10^7 CFU/mL). This sCOD:N:P ratio was set as the normal condition for PHB production based on the results from our previous study which found that KKU38 could effectively accumulate PHB at this sCOD:N:P by using cassava starch hydrolysate as a carbon source. Flasks were incubated at 30°C and shaken at 150 rpm for 7 days. Cultured medium were taken every 24 h to determine the biomass, PHB and sCOD concentrations. The PHB production from CSW and FCSW without inoculation of KKU38 was parallel conducted as control.

In PHB producing microbes, their synthesis take place if a suitable carbon substrate is available in excess and cellular growth is limited by other nutrients like nitrogen or phosphate (Sindhu et al. 2011). As the result indicated that non-diluted FCSW gave the maximum PHB content and PHB yield, it was used in the study on the effect of sCOD:N:P ratios on PHB production. The COD:N:P (100:1:11) is a normal condition which it derived from the comparison of the composition of E2 medium. When the COD value is 100, value of N and P is the ratio of 100:1:11. N is more than 1, called it the N-excess, N is less than 1, called it the N-limited in the same case with P.

The effects of three operational factors, i.e. sCOD, nitrogen and phosphorus by mean of sCOD:N:P ratio variation of 100:0.5:11 (N-limited), 100:10:11 (N-excess), 100:1:1 (P-limited), 100:1:20 (P-excess), 100: 0.5:1 (N&P-limited) and 100:10:20 (N&P-excess) on PHB production and sCOD removal were examined. Batch experiment was carried out in the similar manners of the study on the effect of substrate concentration described above.

TABLE 1. Chemical characteristics of CSW and FCSW

Parameter	CSW	FCSW
pH	4.60	5.30
Total Chemical Oxygen Demand, tCOD (mg/L)	19,854	17,200
Soluble COD, sCOD (mg/L)	12,500	12,400
Total nitrogen (mg/L)	1,410	71.74
Total phosphorus (mg/L)	3,300	81.11
Acetic acid (mg/L)	1.62	31.03
Butyric acid (mg/L)	0.35	16.30
Propionic acid (mg/L)	0.09	16.30

ANALYTICAL PROCEDURES

Cell growth was monitored by measuring the absorbance at 600 nm with Optima SP300 spectrophotometer (Optima, Japan). Cell mass concentration was determined by standard calibration curve between OD_{600} and cell dry weight (CDW), was determined by determination of dry weight (Jung et al. 2000). Culture sample (1 mL) was centrifuged (12,000 g, 10 min, 4°C) and the cell pellet was washed in deionized water, centrifuged and dried to constant weight (105°C, 24 h).

The quantitative of PHB was determined by gravimetric method (modified from Kemavongse et al. 2008). A sodium hypochlorite solution was added to the biomass pellet. The mixture was incubated on an orbital shaker (60 min, 200 rpm, 37°C). The solids were recovered by centrifugation (12,000g, 10 min, 4°C) and washed with 1 mL of acetone after that centrifuged again (12,000 g, 10 min, 4°C). The pellet washed with 1 mL of sterile water and centrifuged again (12,000 g, 10 min, 4°C). Finally, chloroform was added to the pellet and dried (90°C, 24 h) to constant weight in reweighed tube. The PHB content relative to biomass was calculated as the mass of PHB obtained per unit dry cell weight. The residual cell concentration was calculated by subtract the biomass concentration (g/L) with the PHB concentration (g/L) at the same sampling time. Total COD and sCOD concentrations were analyzed by using the dichromate closed-reflux method.

The concentrations of volatile fatty acids (VFAs) including acetic (HAc), propionic (HPr) and normal butyric (HBu) acids were first centrifuged at 10,000 rpm for 10 min, acidified by 0.2 N oxalic acid and filtered through 0.45 μm cellulose acetate membrane. The same GC model with a flame ionization detector (FID) and a 30 m × 0.25 mm × 0.25 μm capillary column (Stabiwax) was used. The temperature 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 carrier gas with a flow rate of 66 mL/min.

RESULTS AND DISCUSSION

EFFECT OF SUBSTRATE CONCENTRATION ON PHB PRODUCTION

The effects of CSW and FCSW concentrations on PHB production were investigated. The PHB accumulation by normal flora presence in the CSW and FCSW, without inoculation of *Cupriavidus* sp. KKU38, was negligible (data not shown) suggesting that KKU38 played an important role on PHB production in this study. The parameters of PHB production obtained from the treatments inoculated with KKU38 were summarized in Table 2. The results indicated that PHB and biomass production trended to increase with the increase in substrate concentration (Table 2). The high concentration of CSW and FCSW up to 100% (approximately 12,500 mg COD/L) did not show the inhibitory effect on PHB and biomass production by *Cupriavidus* sp. KKU38. The PHB content values trended to be greater when the FCSW was used as substrate in comparison to CSW. This might be due to the fact that fermentation process could convert the organic materials in the CSW to be VFAs i.e., butyric and acetic acids which could be easier to be used by KKU38 to produce PHB. The volatile fatty acids could be utilized to produce PHAs by various types of PHA producers such as *Alcaligenes eutorphus* (Yu 2001) and *Ralstonia eutropha* (Du et al. 2001). *A. eutorphus* produce PHB from butyric acid while poly(hydroxybutyrate-hydroxyvalerate) was polymerized from propionic acid when the effluent from hydrogen fermentation of starch wastewater was used as substrate (Yu 2001). The maximum PHB content of 80.52% and PHB yield of 0.13 g PHB/g sCOD consumed were obtained when non diluted FCSW was used (Table 2), therefore the non diluted FCSW was used in the further experiment to study the effect of nitrogen and phosphorus concentrations on PHB production by KKU38.

The relatively high percentage of sCOD removal of approximately 81.45% could be obtained at the end of PHB production which could be a positive effect when discharging the effluent to the environment.

TABLE 2. PHB production and sCOD removal at the different initial concentrations of CSW and FCSW

CSW or FCSW conc.	sCOD remaining at day 7 (g/L)		sCOD consumption at day 7 (g/L)		sCOD removal (%)		Maximum cell concentration (g/L)		Maximum PHB production (g/L)		Maximum PHB content (%)		Maximum PHB yield (g PHB/g sCOD consumed)	
	CSW	FSCW	CSW	FCSW	CSW	FCSW	CSW	FCSW	CSW	FCSW	CSW	FCSW	CSW	FCSW
20%	1.013	0.18	1.17	1.12	53.60	86.15	1.16	1.45	0.13	0.15	38.23	53.98	0.11	0.12
40%	0.47	1.13	2.73	2.09	85.31	64.91	1.11	1.34	0.23	0.55	56.61	74.46	0.08	0.11
60%	0.49	0.2	4.85	5.29	90.82	96.36	1.86	1.85	0.31	0.57	17.99	31.50	0.12	0.12
80%	1.43	2.09	8.96	9.76	86.24	82.36	2.41	1.83	0.41	2.43	39.24	36.47	0.05	0.07
100%	2.09	2.2	10.35	12.99	83.20	85.52	1.49	2.67	0.68	1.57	64.29	80.52	0.07	0.13

EFFECT OF SCOD:N:P RATIO ON PHB PRODUCTION

The effects of nitrogen and phosphorus concentrations by mean of sCOD:N:P ratios variation on PHB production from non-diluted FCSW by *Cupriavidus* sp. KKU38 were investigated. The example of PHB and biomass production and sCOD reduction profiles during cultivation of *Capriavidus* sp. KKU38 using non-diluted FCSW as substrate at N-limited condition (sCOD:N:P ratio of 100:0.5:11) are shown in Figure 1. The sCOD concentration decreased with the increased in biomass and PHB concentration which indicated that the organic matters in the FCSW were consumed to produce biomass and PHB by KKU38. At the exponential phase of growth, cell residual concentration was observed to be stable while the PHB content increased overtime (Figure 1). These results implied that at the sCOD:N:P ratio of 100:0.5:11, KKU38 prefers to accumulate the organic matters into the cell and polymerized to be PHB instead of using the organic matters for microbial growth.

The parameters obtained from batch PHB production at different sCOD:N:P ratios are summarized in Table 3. PHB accumulation occurred in all the experiments with the PHB contents of greater than 59% (Table 3) which was higher than that of previously reported for the PHB production using the acidogenic effluent from the fermentation of real wastewater (Bengtsson et al. 2008; Yu 2001). The excess of nutrients for both N and P-sources facilitated the growth of KKU38, but worsend the PHB accumulation (Table 3). The limitation of N-source (sCOD:N:P ratio of 100:0.5:1) was the optimal condition for PHB production giving the maximum PHB content and yield of 85.53% and 0.31 g PHB/g sCOD consumed, respectively. This observed PHB yield coincided with the findings of Dionisi et al. (2008) and Bengtsson et al.(2008) on the use of VFA mixtures presence in the fermented broth as substrate.

The relatively high sCOD removal efficiency of 73.82% at N-limited condition demonstrated the concept of water quality improvements alongside with production of a value-added by-product as PHB. The remained total sCOD of 26.18% might be the non-organic matters or which were not suitable for microbial growth and PHB accumulation. Over the entire 2 steps, PHB production by using the obtained optimum condition exhibited a potential to produce 0.19 kg of PHB per kg of sCOD containing in cassava starch manufacturing wastewater as shown in Figure 2.

CONCLUSION

The PHB production from 12,500 mg-sCOD/L fermented cassava starch manufacturing wastewater by *Cupriavidus* sp. KKU38 was optimized at the soluble COD:N:P ratio of 100:0.5:11 which gave the maximum PHB content and yield of 85.53% and 0.31 g PHB/g COD consumption, respectively. By using the proposed PHB production process, a potential to produce 0.19 kg of PHB from 1 kg of sCOD containing in cassava starch manufacturing wastewater was exhibited. The relatively high sCOD removal efficiency of 73.82% at the optimal condition could be obtained which demonstrated the concept of water quality improvements alongside with production of a value-added by-product as PHB.

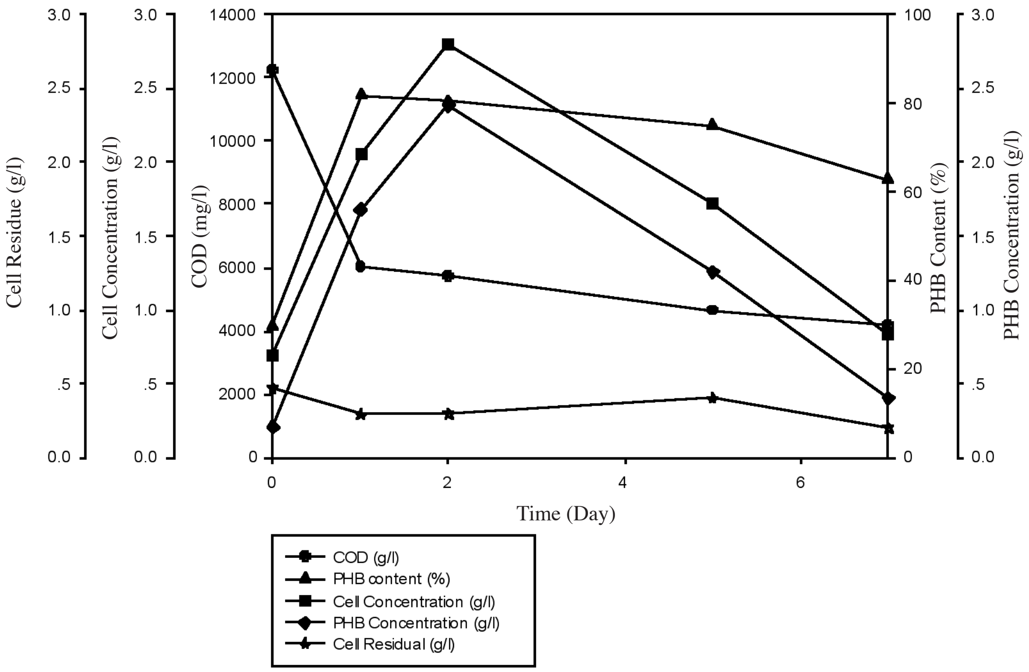

FIGURE 1. Overall efficiency of the process in conversion of 1 kg of sCOD from cassava starch manufacturing wastewater into PHB

TABLE 3. PHB production by *Cupriavidus* sp. KKU38 from non-diluted FCSW at various sCOD:N:P ratios

Sample	sCOD:N:P ratio	sCOD remaining at day 7 (g/L)	sCOD consumption at day 7 (g/L)	sCOD removal	Maximum cell concentration (g/L)	Maximum PHB production (g/L)	Maximum PHB content (%)	Maximum PHB yield (g PHB/g sCOD consumption)
A (Normal)	100:1:11	2.2	12.99	85.52	2.67±0.26	1.57±0.06	80.52±3.59	0.13
B (N-limited)	100:0.5:11	2.72	7.67	73.82	2.80±0.16	2.39±0.01	85.53±4.93	0.31
C (N-excess)	100:10:11	5.97	7.07	54.22	3.58±0.06	2.00±0.13	72.97±4.47	0.28
D (P-limited)	100:1:1	3.81	8.91	70.05	3.18±0.28	2.26±0.08	76.22±8.44	0.25
E (P-excess)	100:1:20	2.96	10.24	77.58	3.48±0.23	2.02±0.12	59.50±5.21	0.20
F (N&P-limited)	100:0.5:1	3.13	9.15	74.51	3.47±0.16	2.05±0.05	61.82±6.64	0.22
G (N&P-excess)	100:10:20	2.11	10.05	82.65	3.80±0.17	2.27±0.05	60.37±4.32	0.23

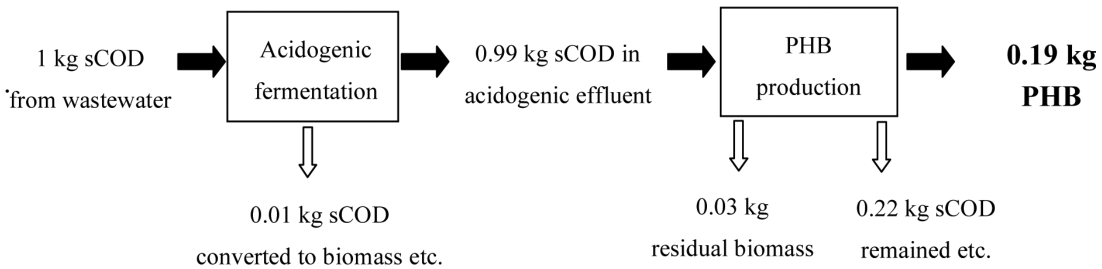

FIGURE 2. Overall efficiency of the process in conversion of 1 kg of sCOD from cassava starch manufacturing wastewater into PHB

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S. Sangyoka*
Program in Environmental Science
Faculty of Science and Technology
Phibulsongkram Rajabhat University
Phitsanulok
65000 Thailand

N. Poomipuk
Department of Biotechnology
Faculty of Technology
Khon Kaen University
Khon Kaen
40000 Thailand

A. Reungsang
Fermentation Research Center for Value Added Agricultural Products
Faculty of Technology
Khon Kaen University
Khon Kaen
40002 Thailand

*Corresponding author; email: suksaman@hotmail.com

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