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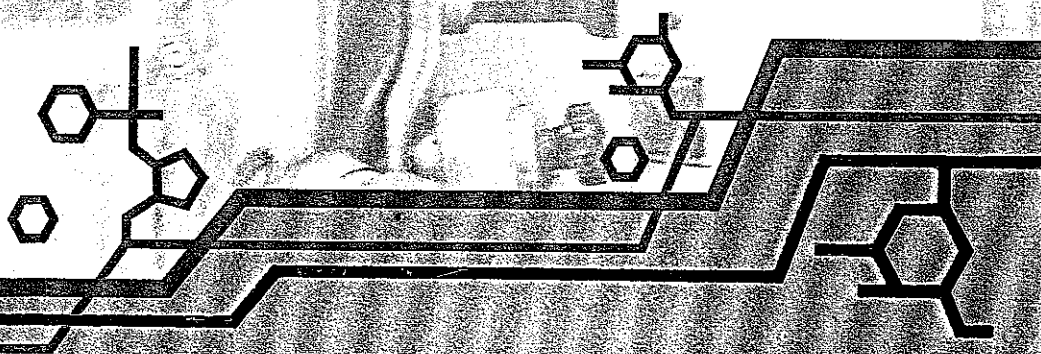
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Optimum conditions for a production of Polyhydroxybutyrate from cassava wastewater by a newly isolated *Cupriavidus* sp. KKU38

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Abstract

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 firstly conducted to obtain the volatile fatty acids (VFAs) which were more efficient to use for PHB production as compared to raw CSW. The effect for substrate concentration and nutrients, i.e. nitrogen and phosphorus concentrations by mean of COD:N:P ratio variation were 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 which 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, a potential to produce 0.19 kg of PHB from 1.0 kg of sCOD containing in cassava starch manufacturing wastewater was exhibited. The relatively high COD 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.

Keywords: polyhydroxybutyrate (PHB), cassava wastewater, *Cupriavidus* sp. KKU38

Introduction

Plastics materials have become an integral part of contemporary life due to their versatile qualities of lightness, durability and resistance to degradation. These nondegradable plastic accumulate in the environment at a rate of millions of ton per year causing several problems. Recently, issues concerning the global environment and solid waste management have created much interest in the development of biodegradable plastics (Anderson and Dawes, 1990). Poly- β -hydroxyl butyrate (PHB) is the best characterized of all 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 remarkable similar to polypropylene and polyethylene. It can be easily 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, the techniques for cost reduction such as strain development, cultivation and downstream process strategy improvement and searching of inexpensive substrates for PHB production are required (Choi and Lee, 1999).

In this study, the possibility of using cassava starch manufacturing wastewater (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 the suitable condition for PHB and biomass productions as well as the COD reduction when the CSW was used as a substrate.

Material 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. 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.

Table 1 Chemical characteristics of CSW and FCSW

Parameter	CSW	FCSW
pH	4.60	5.3
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	16.3
Propionic acid (mg/l)	0.09	16.3

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 flask. The flask was incubated at 30 °C and 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

E2 medium (Lageveen et al., 1988) was used as a cultivation medium in this study. It contains (g/l in distilled water): $\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$ 3.5; $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ 7.5; KH_2PO_4 3.7; 100 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 10 ml; MT microelements stock solution: 1ml (MT microelements stock solution contained (g/l in 1 N HCl): $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 2.78; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 1.98; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ 2.81; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1.47; $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ 0.17; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.29). The initial pH of E2 medium was 7.0. 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 sCOD concentration in the substrate on PHB production and sCOD removal were firstly investigated. E2 medium contained with the various CSW or FCSW concentrations of 20, 40, 80 and 100% 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.

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 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.

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. The quantitative of PHB was determined by gravimetric method modified from Kemavongse et al. (2008). Briefly, a 10-ml culture medium was centrifuge at 5,000 rpm, 4 °C for 15 min and the cell pellet was washed with 10-ml distilled water. The cell pellet was harvested by centrifugation and dried at 105 °C for 48 h or till constant weight obtained. After determination of dry cell weight, a 10 ml of sodium hypochlorite (0.4% w/v) was added to the dried biomass pellet and mixed well. The mixtures were incubated at 37 °C for 1 h. The solids were recovered by centrifugation at 10,000 rpm for 15 min and washed with 10 ml of acetone. The tube was then centrifuged, washed with 10-ml distilled water and centrifuged again to obtain the pellet part. Ten-ml of chloroform was then added to the pellet and dried at 50 °C for 12 h following with 105 °C for 48 h. The PHB content relative to biomass was calculated as a 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. tCOD and sCOD concentrations were analyzed by using the dichromate closed-reflux method (APHA, 1995).

Results and discussions

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 eutrophus* (Yu 2001) and *Ralstonia eutropha* (Du et al. 2001; Yu et al., 2002). *A. eutrophus* 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 FCS W 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	FCSW	CSW	FCSW	CSW	FCS W	CSW	FCS W	CSW	FCS W	CSW	FCSW	CSW	FCSW
20%	1.013	0.18	1.17	1.12	53.6 0	86.1 5	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	85.3 1	64.9 1	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	90.8 2	96.3 6	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	86.2 4	82.3 6	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	83.2 0	85.5 2	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 Fig. 1. The sCOD concentration decreased with the increased in biomass and PHB concentrations 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 (Fig. 1). These results implied that at the sCOD:N:P ratio of 100:0.5:11, KKU38 prefer 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 were 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. 2004 and Bengtsson et al. 2008 on the use of VFA mixtures presence in the fermented broth as substrate.

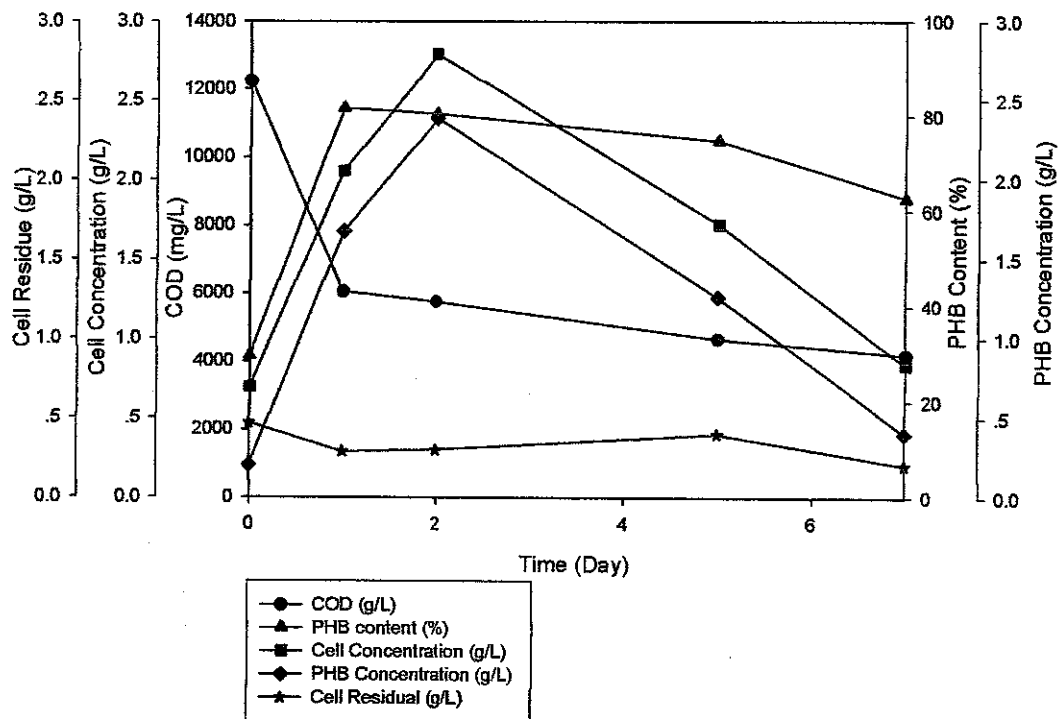


Figure 1 PHB production by *Cupriavidus* sp. KKKU38 from non-diluted FCSW at the N-limited condition (sCOD:N:P ratio of 100:0.5:1)

Table 3 PHB production by *Cupriavidus* sp. KKKU38 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

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 Fig. 2.

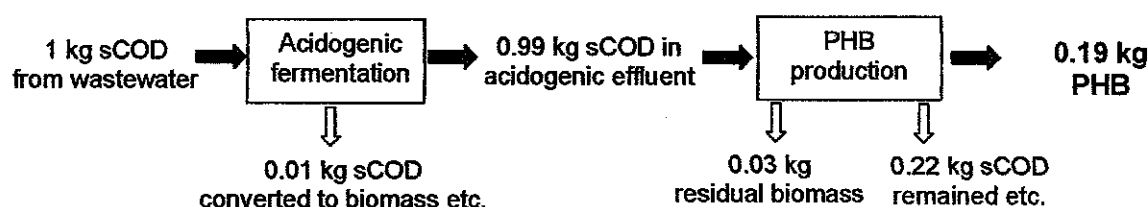


Figure 2 Overall efficiency of the process in conversion of 1 kg of sCOD from cassava starch manufacturing wastewater into PHB.

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 1.0 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.

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