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Yields of Polyhydroxyalkanoates (PHAs) during Batch Fermentation of Sugar Cane Juice by *Alcaligenes latus* and *Alcaligenes eutrophus*

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Abstract: In this work, sugar cane juice was fermented to produce polyhydroxyalkanoates (PHAs) by *Alcaligenes latus* TISTR 1403 and *A. eutrophus* TISTR 1095. The juice was characterized and composed of total sugars 105.5 $g \cdot L^{-1}$ (sucrose 36.6 $g \cdot L^{-1}$, fructose 26.0 $g \cdot L^{-1}$, glucose 21.8 $g \cdot L^{-1}$ and other sugars 21.1 $g \cdot L^{-1}$). Each inoculums (10%, v/v) was separately cultivated in the medium containing 20 $g \cdot L^{-1}$ total sugars under condition (30ºC, 200 rpm, pH 6.5-7). It was found that the *A. eutrophus* can be grown better than the *A. latus*. Only the *A. eutrophus* was further cultured under different total sugar concentrations (20, 30, 40 and 50 $g \cdot L^{-1}$). The optimal contents of total sugar, dry cell mass (DCM) and maximum PHAs were obtained at 50 $g \cdot L^{-1}$, 6.013 $g \cdot L^{-1}$ and 1.84 $g \cdot L^{-1}$, respectively after 60 h fermentation which were converted to biomass yield ($Y_{x/s}$), product yield ($Y_{p/s}$), specific product yield ($Y_{p/x}$) and productivity of 0.163, 0.05, 0.306 and 0.031 $g \cdot L^{-1}h^{-1}$. Large scale of PHAs production was conducted in 5 L fermentor using the optimal condition obtained under 30% dissolved oxygen. The DCM and the maximum PHAs were 5.881 $g \cdot L^{-1}$and 1.281 $g \cdot L^{-1}$ which were calculated to values of $Y_{x/s}$, $Y_{p/s}$, $Y_{p/x}$ and productivity at 0.19, 0.04, 0.218 and 0.021 $g \cdot L^{-1}h^{-1}$, respectively.

Key words: *Alcaligenes latus*, *Alcaligenes eutrophus*, fermentation, PHAs, sugar cane juice.

1. Introduction

Polyhydroxylakanoates (PHAs) are biodegradable, biocompatible, and natural polymers. Typically, they can be produced intracellularly by various microorganisms mostly produced by bacteria as storage material for carbon and energy reserves under unbalanced growth condition such as nutrient limitation [1-5]. Naturally, properties of PHAs are similar to thermoplastic that was obtained from petrochemical industry such as polypropylene (PP) and polyethylene (PE). They also can be produced from agricultural products such as sugar cane, sweet sorghum, molasses and even from palm oil mill effluent as carbon sources by various microorganisms mostly bacterial strains [4-6]. The composition of PHAs depends on the microorganism and nature of the carbon sources allowing the formulation of new polymers with different physicochemical properties as short or mid-chain and long-chain fatty acids. However, the most common forms founded in microorganism cells are polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV). Commercially, production of PHAs costs 10 times more expensive than PE [7]. The important limitations factors in the production of PHAs are special growth condition required, the expensive raw materials such as the

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producer microorganisms and substrate composition, cultures condition, fermentation processes (batch, fed-batch, repeated batch or fed-batch, and continuous modes) and the highest cost of their recovery [1, 8-10].

Alcaligenes eutrophus and *A. latus* are gram-negative bacteria which can be accumulated a large amount of PHAs inside their cells [1, 6, 11] and especially, *A. latus* is the most widely used strain for commercial production of PHAs [12]. It can be utilized many carbon sources [13]. Previous study has reported the optimization of PHB produced by *A. latus* which used sucrose as a carbon source and accumulated up to 63% of dry cell mass after 93 h under optimal condition [11]. Later, *A. latus* was studied to produce PHB by using maple sap which is rich in sucrose. The biomass and PHB concentration were obtained at 4.4 $g \cdot L^{-1}$ and 3.41 $g \cdot L^{-1}$ after 27 h of fermentation [14, 15]. It was proved that the bacteria can be utilized sucrose as a cheaper carbon source than glucose [11, 14-19].

Therefore, this study aims to study the potential use of sugar cane juice as a raw substrate by pure bacterial strains of *Alcaligenes latus* TISTR 1403 and *Alcaligenes eutrophus* TISTR 1095 to produce PHAs via batch fermentation. In addition, the feasibility will be evaluated.

2. Materials and Methods

2.1 Microorganisms and Culture Condition

Pure bacterial strains of *Alcaligenes latus* TISTR 1403 and *A. eutrophus* TISTR 1095 were purchased from Thailand Institute of Science and Technology Research (TISTR) (Bangkok, Thailand). Then, they were maintained on agar slant and Petri dishes on nutrient medium [6]. The inoculums were prepared in nutrient broth under aerobic condition (30ºC, 200 rpm and pH 6.5-7 for 24 h) prior to inoculate into sterile production medium.

2.2 Preparation of Sugar Cane Juice

Sugar cane juice was kindly received from sugar cane industry (Chaiyaphum, Thailand). The juice was kept in refrigerator at -20 ºC prior to use. The juice was thaw and centrifuged to remove other particulates. In addition, the juice was sterilized and then added into the culture medium.

2.3 Batch Fermentation in Flask Scale

Seed inoculums of 10% (v/v) were added into 250 mL flask containing 100 mL modified minimal medium 3 (the prepared method described by Grothe et al.) [11]. One liter of the medium contained: sucrose (20 g), KH_2PO_4 (1.5 g), Na_2HPO_4 (3.57 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $(NH_4)_2SO_4$ (1 g), and trace element solution (1 mL). It should be noted that the concentration of sucrose was replaced by sterilized sugar cane juice used as sole carbon source. The culture was further incubated for 72 hours at 30ºC, 200 rpm and pH 6.5-7.0. In addition, the initial total sugar concentrations at 20, 30 40 and 50 $g \cdot L^{-1}$ were also varied and the medium were collected every 6 h to monitor cell growth, dry cell mass, and total sugar concentration.

2.4 Batch Fermentation in Fermentor

Only, the culture of *A. eutrophus* was then inoculated into a 5-L fermentor with working volume of 2 L using optimal total sugar concentration obtained from the previous flask scale. Fermentation condition was controlled as following: 200 rpm, 2 vvm aeration rate, 30ºC and pH 6.5-7.0. Dissolved oxygen (DO) concentration was monitored with a DO electrode during the whole cultivation and maintained at 30% constant of air saturation by adjusting the aeration rate and agitation rate speed in response to the change of oxygen requirement. In addition, the pH was also monitored with a pH electrode and kept at 6.5-7.0 by adding solutions of sulfuric acid (H_2SO_4) or sodium hydroxide (NaOH) if necessary.

2.5 Analytical Methods

2.5.1 Dry Cell Mass (DCM)

The DCM was determined by the absorbance

method using a spectrophotometer at 600 nm. A 1-mL fermentation broth was centrifuged (×10,000 g, for 10 min) and the cell pellet then was washed for twice with distilled water. The cell was dried at 90ºC until a constant DCM was obtained.

2.5.2 Total Sugar Concentration

Sugar cane juice was measured an initial concentration in term of total sugar using Phenol-Sulfuric method [19] and the juice was also characterized type of sugar contained by HPLC method. In addition, total soluble solid and pH value of the juice were measured by hand refractometer and pH meter.

2.5.3 PHAs Content

After fermentation, the biomass was recovered and enriched using sequencing steps of cell disruption, centrifugation, extraction and evaporation. Finally, PHAs powder was obtained. The powder was dissolved in a conc. sulphuric to determine PHAs content in form of polyhrdroxybutyrate (PHB) by measuring the optical density at 235 nm [20].

3. Results and Discussion

3.1 Growth of A. latus and A. eutrophus

Two bacterial strains of *A. latus* and *A. eutrophus* were cultivated in modified minimal medium with 20 $g \cdot L^{-1}$ initial total sugar concentration in form of the sugar cane juice. Fig. 1 shows the time course of both biomass measured in term of optical density (OD) value. *A. eutrophus* can be obviously grown better than *A. latus* and reached a maximum biomass at 36 h. Previous study reported that *A. latus* can be grown in maple sap which was rich of sucrose [15]. However, in this study, the juice contained of sucrose and glucose in similar amount. It was believed that *A. latus* preferred to use glucose as a primary carbon source instead of sucrose. Then, only *A. eutrophus* was selected to produce PHAs in fermentor.

3.2 Characteristics of Sugar Cane Juice

The juice was measured for its composition. The characterizations of the juice are shown in Table 1.

3.3 Optimal Condition for PHAs Production in Flasks

Initial total sugar concentrations of sugar cane juice were varied at 20, 30, 40 and 50 $g \cdot L^{-1}$ and considered the optimal culture condition Fig. 2 shows the accumulation of PHAs as functions of time. The optimal total sugar concentration of 50 $g \cdot L^{-1}$ was obtained and gave maximum DCM and PHAs at 6.013 $g \cdot L^{-1}$ and 1.84 $g \cdot L^{-1}$ after 60 h. Fig. 3 reveals the time courses of the biomass, the PHA and residual total sugar. When the nitrogen source was exhausted

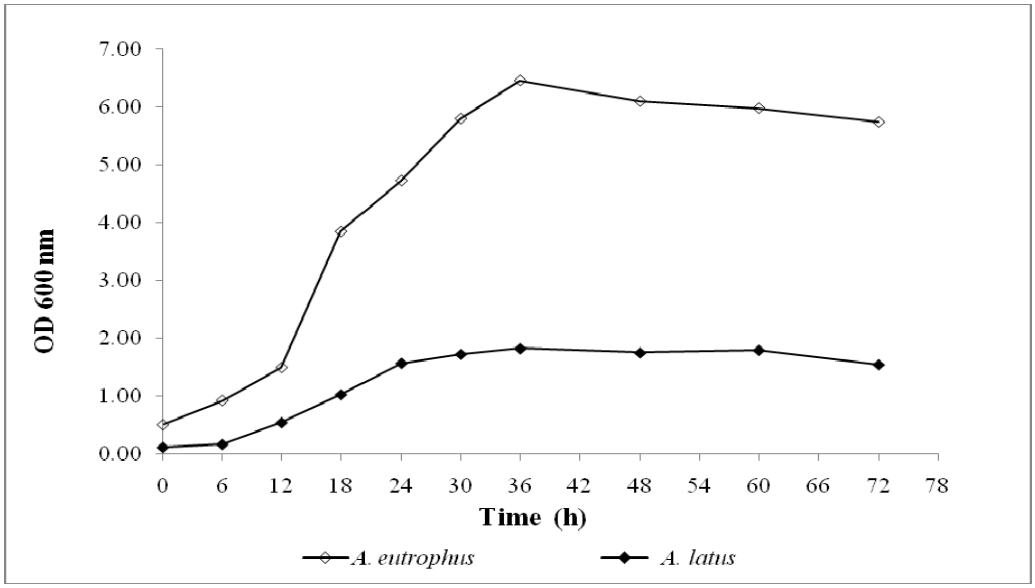

Fig. 1 Growth cultures of *A. latus* TISTR 1403 and *A eutrophus* TISTR 1095 in modified minimal medium.

Table 1 Properties of sugar cane juice (pH 3.4).

Properties	g·L^{-1}
Total sugar	105.5
Sucrose	36.6
Glucose	21.8
Fructose	26.0
Others	21.1
Total soluble solid (Brix)	11.6

completely at t = 60 h, the PHAs were 1.848 g·L^{-1} and the DCM reached a maximum (6.013 g·L^{-1}). The batch fermentation system reached a steady state when t = 72 h. This implied that the start of PHAs accumulation coincides with the exhaustion of nitrogen. As total sugar was increased, the higher content of PHAs was reached. The results obtained were similar to previous studies [3, 6, 13, 21, 22]. However, the total sugar was

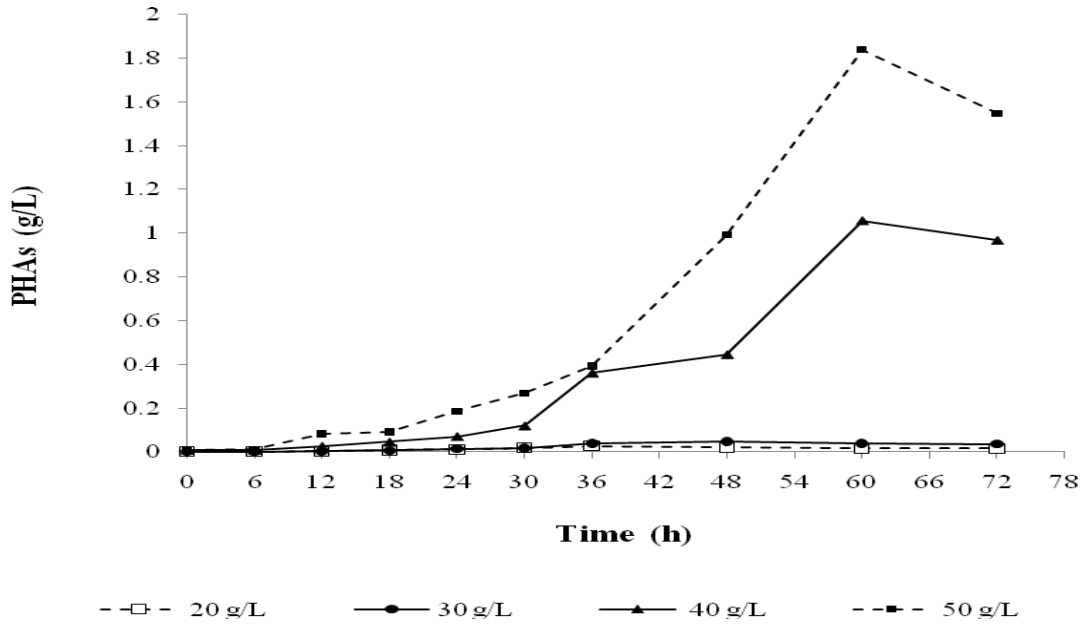

Fig. 2 PHAs content as function of time under variation of total sugar at 20, 30, 40 and 50 g·L^{-1} in shake flask.

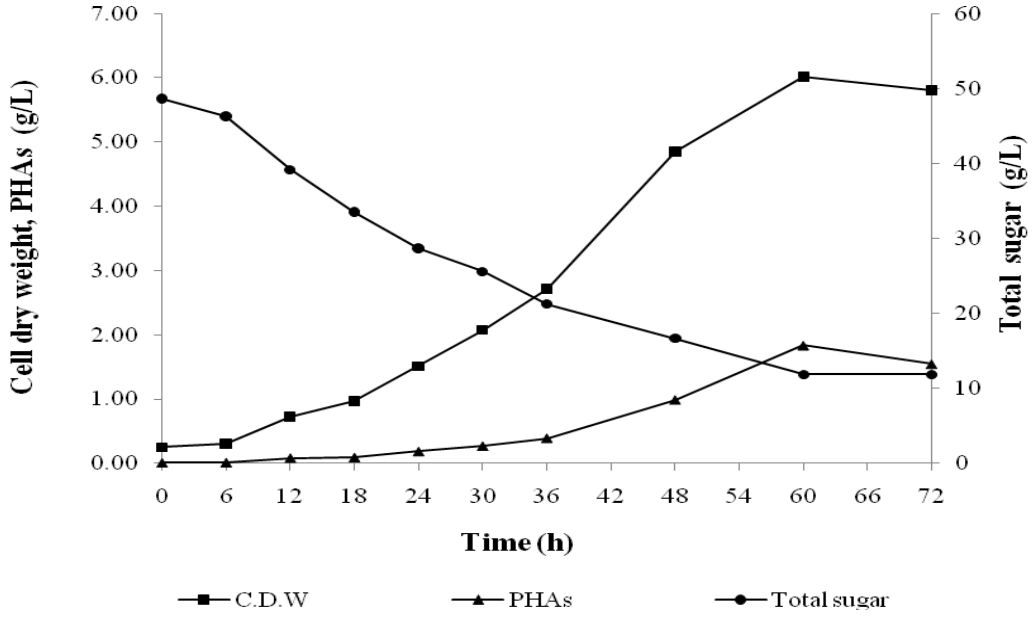

Fig. 3 Profiles of cell dry weight, PHAs and total sugar as functions of time in shake flask.

still remained. Since, it might be the heat during sterilization of the juice caused browning reaction and produced as other compounds and inhibited the growth of bacteria and accumulation of PHAs. In addition, all data were also calculated and represented in terms of biomass yield ($Y_{x/s}$), product yield ($Y_{p/s}$), specific product yield ($Y_{p/x}$) and productivity that were obtained at 0.163, 0.05, 0.306 and 0.031 $g{\cdot}L^{-1}h^{-1}$, respectively. The optimal condition was obtained at 50 $g{\cdot}L^{-1}$ total sugar, 30ºC, 200 rpm and pH 6.5-7.0. The results are summarized in Table 2.

3.4 Production of PHAs in Fermentor

To increase the production of PHAs production, 5-L fermentor with 2-L working volume was considered using the optimal condition obtained from the flask scale. The DCM and the PHAs, and the residue sugar concentration as functions of time are shown in Fig. 4. The batch system reached a steady state with the DCM (5.881 $g{\cdot}L^{-1}$) and the maximum PHAs 1.281 ($g{\cdot}L^{-1}$) at t = 60 h which were calculated to values of $Y_{x/s}$, $Y_{p/s}$, $Y_{p/x}$ and productivity at about 0.190, 0.041, 0.218 and 0.0213 $g{\cdot}L^{-1}h^{-1}$, respectively (Table 3). It seems that the system reached a steady state after 72 h. While the residue total sugar concentration was still remained at about 20 $g{\cdot}L^{-1}$, it seems that the sugar cane juice might contain other composition that was inhibited for the growth of microorganism, although these results were agreed with previous studies [3, 6, 13, 21, 22]. However, all of them used pure primary carbon sources such as glucose, sucrose and secondary carbon source such as propionate etc. On the other hand, in the present study, we have used mixed sugars naturally found in the sugar cane juice. An attempt has undertaken in another juice obtained from agricultural product of sweet sorghum [6]. However, the PHAs yield and their productivity were still low. This may implies that the time for fermentation, an efficient fermentation process and/or recombinant microbial strain needs to improve the production of PHAs.

Table 2 Biomass and PHAs production in different total sugar concentrations.

Total sugar concentrations ($g{\cdot}L^{-1}$)	Biomass ($g{\cdot}L^{-1}$)	PHAs ($g{\cdot}L^{-1}$)	Biomass yield, $Y_{x/s}$	Product yield, $Y_{p/s}$	Specific product yield, $Y_{p/x}$	Productivity ($g{\cdot}L^{-1}h^{-1}$)
20	1.8994	0.0273	0.1241	0.0018	0.0145	0.0008
30	2.1611	0.0480	0.1073	0.0024	0.0224	0.0010
40	3.9988	0.9681	0.1238	0.0230	0.1858	0.0134
50	6.0129	1.8377	0.1634	0.0499	0.3056	0.0306

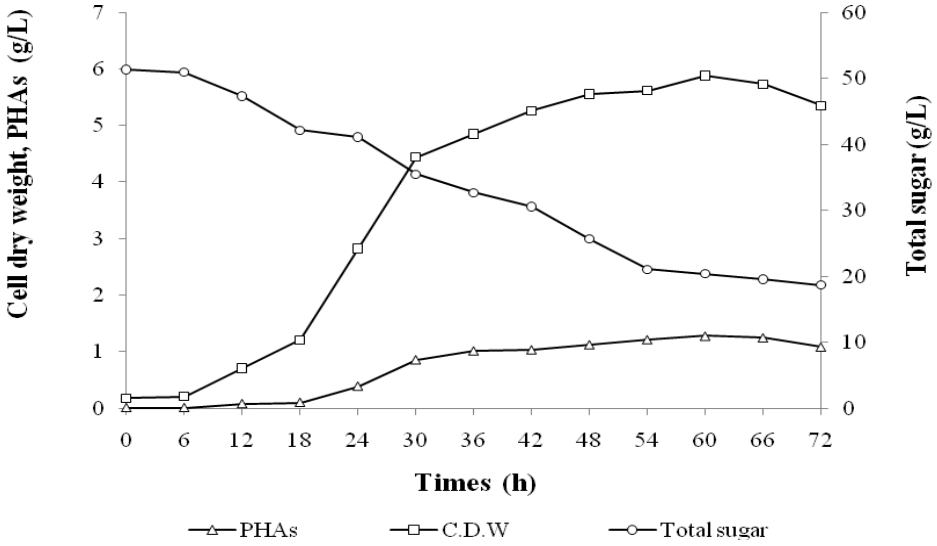

Fig. 4 Profiles of cell dry weight, PHAs, and total sugar as functions of time in fermentor.

Table 3 The production of PHAs in various carbon sources.

Parameters	Bacterial strains				
	A. eutrophus	*A. latus*	*A. eutrophus*	*A. eutrophus*	*A. eutrophus*
Carbon source	Glucose	Sucrose	Sweet sorghum juice	Sugar cane juice	Sugar cane juice
Culture time	50	28.45	48	60	60
Cell concentration ($g \cdot L^{-1}$)	164	143	13.40	5.88	6.01
PHB concentration, ($g \cdot L^{-1}$)	121	71.4	6.87	1.28	1.84
Biomass yield ($Y_{x/s}$)	-	-	0.53	0.197	0.163
PHB yield ($Y_{p/s}$)	0.3	0.17	0.27	0.04	0.05
PHB/dry cell weight (%)	76	50	51.3	21.8	30.6
PHB productivity ($g \cdot L^{-1}h^{-1}$)	2.42	2.5	0.143	0.021	0.031
References	[21]	[23]	[6]	Current study (fermentor)	Current study (flask)

4. Conclusions

The present study shows that *A. eutrophus* can be grown in the medium containing mixed sugars mostly with sucrose from sugarcane juice. The optimal 50 $g \cdot L^{-1}$ total sugar concentration in the juice for PHAs production was obtained in both flask and fermentor and gave the maximum PHAs, $Y_{x/s}$, $Y_{p/s}$ and $Y_{p/x}$. The results obtained clearly showed that agricultural based sugar raw material of sugar cane syrup has a potential use as a cheap carbon source for the production of PHAs in a large scale. For further, response surface methodology (RSM) will be completed for process optimization of the production PHAs via batch fermentation.

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