EFFECT OF GLUCOSE ON PHB PRODUCTION USING *Alcaligenes eutrophus* DSM 545 AND TISTR 1095

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Abstract

Poly-β-hydroxybutyrate (PHB) is biodegradable plastics have received increased attention due to its properties that resemble those of petroleum-based plastics such as polyethylene. PHB could be accumulated within bacterial cells varying glucose concentration in the present study. The concentrations of 5, 10, 15 and 20 g L^{-1} glucose were employed as a single carbon source for the PHB production which compared between *Alcaligenes eutrophus* DSM 545 and *A. eutrophus* TISTR 1095. In shake flask culture, the maximum PHB concentrations were 9.437 g L^{-1} with 10 g L^{-1} glucose, and 1.679 g L^{-1} with 15 g L^{-1} glucose for *A. eutrophus* DSM 545 and *A. eutrophus* TISTR 1095, respectively. The highest PHB yield of 0.321 g PHB g^{-1} glucose was obtained from using 5 g L^{-1}glucose by *A. eutrophus* TISTR 1095. For *A. eutrophus* DSM 545, its highest PHB yield of 0.998 g PHB per g glucose was attainable from 10 g L^{-1} glucose. In addition, the production yield of PHB from *A. eutrophus* DSM 545 decreased against the glucose concentration increased from 15 to 20 g L^{-1}. As a result, the initial concentration of glucose beyond bacterial growth inhibition was appropriately found at 10 g L^{-1}. Further work would go for scale up of PHB production using *A. eutrophus* DSM 545.

Keywords: Poly-β-hydroxybutyrate (PHB), glucose concentration, *A. eutrophus* DSM 545, *A. eutrophus* TISTR 1095

Introduction

Plastic wastes are the global proportions which construct the environmental pollutions. Due to these conventional plastics derived from petroleum processes, they cannot be readily biodegradable. There are several families of biodegradable plastics such as poly-hydroxy alkanoates (PHAs), and their co-polymeric derivatives have been attracted due to their complete biodegradability (Kumar *et al*., 2004). Many microbial strains accumulate PHAs within their cells for using as carbon and energy sources. *Alcaligenes eutrophus* produced PHAs under the imbalance of nutrients and oxygen conditions (Yinguang *et al*., 2003). Furthermore, the limited nutrient and oxygen conditions were studied to increase the productivity of PHAs (Giannis *et al*., 2011). In addition to the ability of biodegradation,

PHAs are biocompatible thermoplastic in nature and possess physical properties similar to the petroleum-derived polymers (Mohd *et al.*, 2012).

Poly-β-hydroxybutyrate (PHB) is a homopolymer which known as a member of PHAs family. It has a high degree of crystallinity and the characteristics of PHB are similar with polypropylene, petro-chemically product (Hao *et al.*, 2010). PHB was produced from microorganisms and stored in cells for granules forming, also PHB has shown the ability of biodegradable material environmentally friendly (Kumar *et al*., 2004). There are many applications of PHB for using in the industries such as it can be used in medicine and soft tissue implants, pharmacy food packaging and agriculture materials (Fereshteh *et al.*, 2012). However, the commercialization of PHB production has still used a higher production cost compared with petrochemical-based plastic materials. Due to the recovery step of PHB from microorganisms needs to put the high value of the equipment on its production costs. Therefore, several methods have been suggested to use for recovery of PHB such as organic solvent extraction, enzyme digestion, sodium hypochlorite digestion, sodium hypochlorite digestion, chloroform extraction and mechanical methods (Yinguang *et al*., 2003). Moreover, the improvement of the fermentation process for PHB production was included to study. Numerous studies have been carried out on the feeding of glucose and an organic acid to achieve a high cell density and high productivity. After testing, several glucose feeding conditions have pointed out that the concentration of glucose from 10 to 25 g L^{-1} was important for high productivities. However, in all of them, the controlled glucose concentrations have still varied widely and needed more supporting of knowledges from the research for development further (Longan *et al*., 2003).

In this study, glucose was used as the sole carbon source for *Alcaligenes eutrophus* DSM 545 (Sirisansaneeyakul and Mahasubpaiboon, 2003) and *A. eutrophus* TISTR 1095 in shake flask cultures for the production of PHB by variation of glucose concentration. The fermentation kinetics was elucidated in comparison for their PHB accumulation.

Methodology

Bacterial strains and inoculum preparation

Alcaligenes eutrophus DSM 545 and *A. eutrophus* TISTR 1095 were prepared as inoculum in two steps. The stock culture of *A. eutrophus* DSM 545 and *A. eutrophus* TISTR 1095 were first streaked on nutrient agar and incubated at 30°C for 24 h for making the pure culture. Then several colonies of *A. eutrophus* DSM 545 and *A. eutrophus* TISTR 1095 were used to inoculate into a 500-mL Erlenmeyer flask containing 150 mL of sterilized nutrient broth medium and incubated at 30°C with an agitation rate of 250 rpm for 24 h.

Cultivation medium

A. eutrophus DSM 545 and *A. eutrophus* TISTR 1095 were cultivated in the modified medium as described by Lee *et al*. (1994). For PHB production medium 1 L was described as follows: $(NH_4)_2SO_4$ 1.2 g, KH_2PO_4 13.3 g, $MgSO_4.7H_2O$ 1.2 g, citric acid 1.7 g, trace element solution 10 mL and adjusted to pH 6.8. Trace element was prepared as follows:

$FeSO_4.7H_2O$ 10 g, $ZnSO_4.7H_2O$ 2.25 g, $CuSO_4.5H_2O$ 1 g, $MnSO_4.5H_2O$ 0.5 g, $CaCl_2.2H_2O$ 2 g, $Na_2B_4O_7.10H_2O$ 0.23 g, $(NH_4)_6Mo_7O_{24}$ 0.1 g and 36% of HCl 10 mL. The concentrations of glucose were varied at 5, 10, 15 and 20 g L^{-1}.

Culture condition in shake flask culture

The inoculum as 10% v/v was used to inoculate into a 1,000-mL Erlenmeyer flask containing 300 mL of sterile PHB production medium. Then all of cultures were incubated at 30°C with an agitation rate of 250 rpm for 48 h and samplings were taken for analysis every 4 h.

Glucose, biomass and PHB determination

Cell growth was analyzed by measuring the absorbance at 650 nm. Broth samples were collected and centrifuged. The supernatants were analyzed for the consumption of glucose. The quantity of glucose was measured by using DNS method (Saqib and Whitney, 2011). The biomass were washed twice with water to remove residual culture medium and used for analysis of PHB. For the biopolymer quantification, PHB contents produced from *A. eutrophus* DSM 545 and *A. eutrophus* TISTR 1095 were determined as described by Law and Slepecky (1960 and 1961). Briefly, dry biomass was resuspended in sodium hypochlorite solution. After incubation for 1 h at 37^{o}C, the lipid granules were centrifuged and washed with water, acetone and ethanol, respectively. Finally, the dissolved polymer was extracted by chloroform and boiling for 2 min. The mixture solution (dissolved polymer and chloroform) was filtered and then the filtrate was used for PHB assay. Quantity of PHB assay, 10 mL of concentrated sulfuric acid was added into the PHB solution and heated for 10 min at 100^{o}C. After that the solution was cooled, the absorbance at 235 nm was measured against with the PHB standard curve by using sulfuric acid as the blank.

Results

Production of poly-β-hydroxybutyrate (PHB)

From the experiments, the cell growth profile of *A. eutrophus* DSM 545 for the production of PHB in glucose-based medium was shown in Table 1. The maximum quantity of PHB reached 3.621, 9.437, 2.845 and 0.919 g L^{-1} from 5, 10, 15 and 20 g L^{-1} glucose used in the PHB production medium, respectively. From the result, it showed that the highest yield of PHB reached 0.998 g PHB per g glucose with using an initial 10 g L^{-1} glucose as carbon source, which was greater than those with using 5, 15 and 20 g L^{-1} glucose.

The profile for cell growth, substrate consumption and PHB production in shake flask culture of *A. eutrophus* DSM 545 was shown in Figure 1. The results showed that an increase of cell growth increased the production of PHB, while glucose as substrate was consumed and decreased as a function of incubation times. The phase of cell growth found clearly during 12-16 h of cultivation time and kept further constant in its stationary phase. However, the PHB production increased gradually and rapidly after 32 h of the cultivation. The glucose concentration decreased and exhausted in the stationary growth phase after 16 h of the cultivation. In comparison among experiments, for the lower concentration of 5 and 10 g L^{-1}

glucose, glucose was consumed better than those with using higher initial concentration of 15 and 20 g L^{-1} glucose. These indicated that there was the substrate inhibition in shake flask culture of *A. eutrophus* DSM 545 for the PHB production.

Table 1 Production of PHB from *A. eutrophus* DSM 545 and TISTR 1095

Strain	Glucose (g L^{-1})	ΔS^a (g L^{-1})	$PHB_{max}{}^b$ (g L^{-1})	$Y_{P/S}{}^c$ (g PHB per g glucose)	$Q_p{}^d$ (g L^{-1} h^{-1})	% Consumption
A. eutrophus DSM 545	5	4.350	3.621	0.832	0.075	87.00
	10	9.456	9.437	0.998	0.214	94.56
	15	7.591	2.845	0.375	0.059	50.61
	20	7.993	0.919	0.115	0.026	39.97
A. eutrophus TISTR 1095	5	4.591	1.476	0.321	0.031	91.82
	10	9.006	0.870	0.097	0.018	90.06
	15	7.673	1.679	0.219	0.035	51.15
	20	7.929	1.325	0.167	0.030	39.65

[a] The amount of glucose consumed after 48 h incubation
[b] The maximum concentration of PHB
[c] The yield of PHB based on glucose consumption
[d] The volumetric productivity of PHB

For *A. eutrophus* TISTR 1095, its profile for growth and the production of PHB in glucose-based medium was shown in Table 1. The maximum quantity of PHB reached 1.476, 0.870 1.679 and 1.325 g L^{-1} from initial 5, 10, 15 and 20 g L^{-1} glucose contained in PHB production medium, respectively. Moreover, the highest yield of PHB reached 0.321 g PHB per g glucose with using 5 g L^{-1} glucose, which was greater than those with using 10, 15 and 20 g L^{-1} glucose.

The profile for cell growth, substrate consumption and PHB production in shake flask culture of *A. eutrophus* TISTR 1095 was shown in Figure 2. Similarly, *A. eutrophus* TISTR 1095 found its growth, the PHB production and the glucose consumption were clearly due to incubation time. The cell growth increased gradually after inoculation and kept constant in stationary phase after 12 h of the cultivation. Clearly, the production PHB increased continuously with the consumption of glucose. Among various initial concentrations of glucose, the lower concentration of 5 and 10 g L^{-1} glucose found better consumed rather than those higher concentration of 15 and 20 g L^{-1} glucose. These results showed the similar influence of glucose concentration on growths of *A. eutrophus* TISTR 1095 and *A. eutrophus* DSM 545 for the production of PHB.

Discussion and Conclusion

Poly-β-hydroxybutyrate (PHB) was produced from glucose and accumulated intracellularly by *A. eutrophus* DSM 545 and *A. eutrophus* TISTR 1095. Glucose was used as a sole carbon source in this study for PHB production. Under the appropriate condition, *A. eutrophus* DSM 545 and *A. eutrophus* TISTR 1095 produced PHB with the highest of 9.437 and 1.679 g L^{-1}, respectively. Moreover, the influence of glucose concentration on the PHB production by *A. eutrophus* DSM 545 and *A. eutrophus* TISTR 1095 was clearly shown in this study. The higher $Y_{P/S}$ was obtainable when glucose maintained at 10 g L^{-1} from *A. eutrophus* DSM 545, and its PHB yield lowered with glucose higher than this concentration. This explained that under the limiting nutrient conditions cells may shift its protein synthesis to PHB synthesis for survival (Kumar *et al*., 2004). Limitation of carbon source may cause a slow-down in the tricarboxylic acid cycle, and a decrease in the free coenzyme-A concentration. As compared to *A. eutrophus* TISTR 1095, the production of PHB was observed more superior with *A. eutrophus* DSM 545, whose PHB accumulated intracellularly higher than *A. eutrophus* TISTR 1095. On the other hand, *A. eutrophus* TISTR 1095 produced PHB accordingly based on the concentration of glucose. However, *A. eutrophus* TISTR 1095 consumed glucose clearly for bacterial cell growth better than that for PHB production. The strain is found not attractive for use as potential strain for the production of PHB, as compared to *A. eutrophus* DSM 545 (Table 1). Therefore, *A. eutrophus* DSM 545 will be further used for optimizing the production of PHB at the fermenter level. This preliminary work gives potentially the process development for the production of PHB using *A. eutrophus* DSM 545 under optimal glucose concentration, in which cost reduction must be considered basically from glucose used as a sole carbon.

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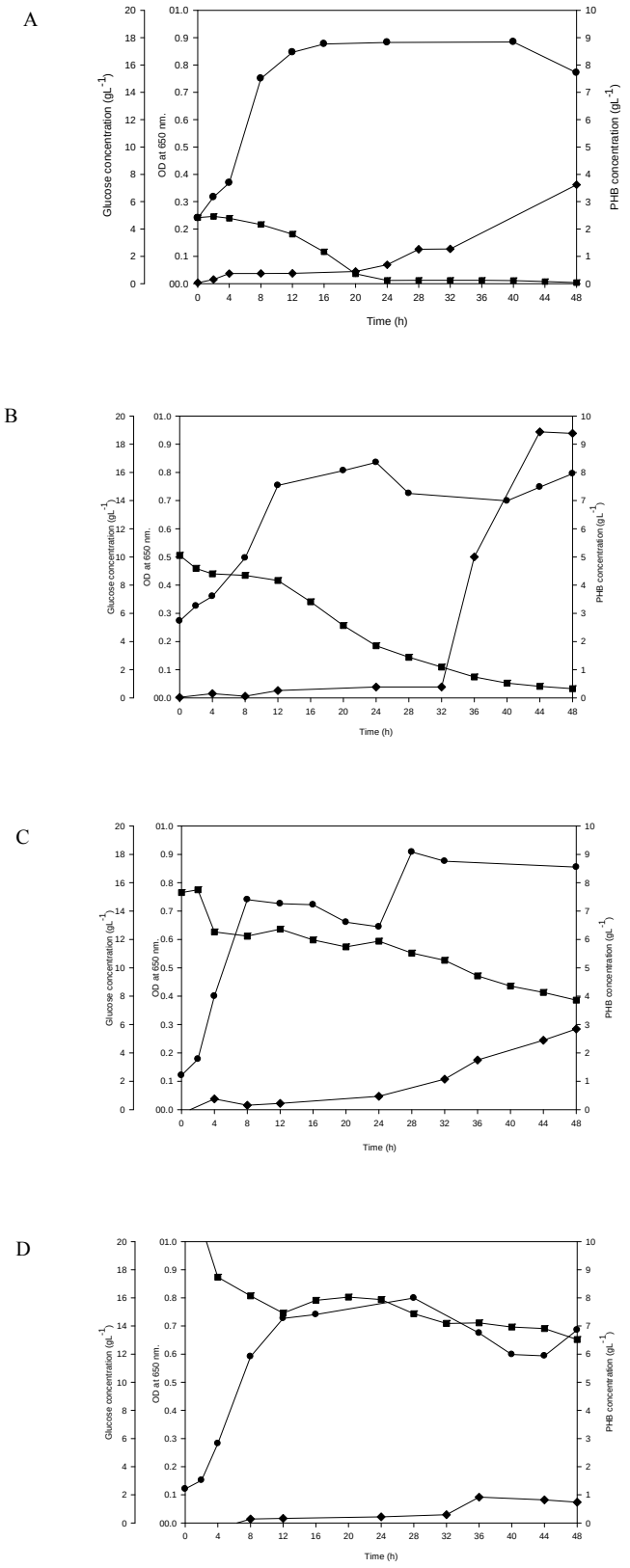

Figure 1 The growth and production of PHB by *A. eutrophus* DSM 545 in shake culture. Changes of cell growth (●), glucose (■) and PHB (◆) concentrations during cultivation at various glucose concentrations: (A) 5 g L^{-1}, (B) 10 g L^{-1}, (C) 15 g L^{-1} and (D) 20 g L^{-1}.

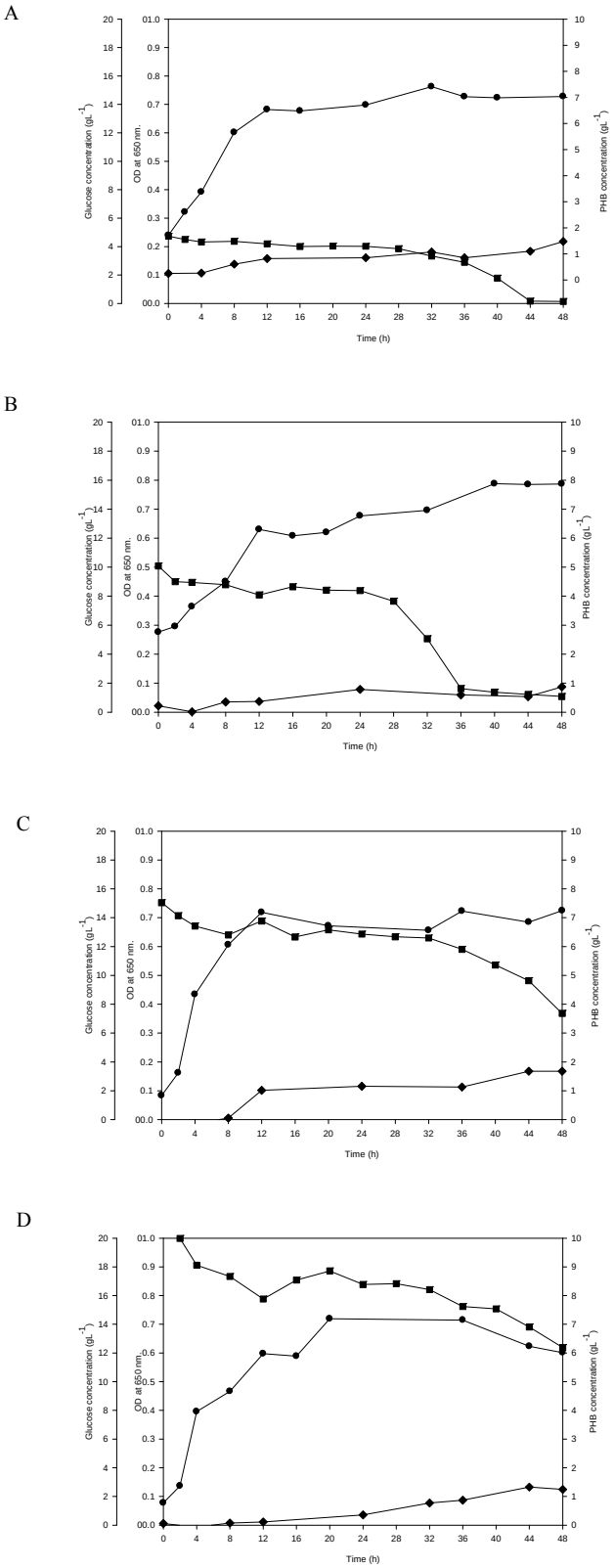

Figure 2 The growth and production of PHB by *A. eutrophus* TISTR 1095 in shake culture. Changes of cell growth (●), glucose (■) and PHB (◆) concentrations during cultivation at various glucose concentrations: (A) 5 g L^{-1}, (B) 10 g L^{-1}, (C) 15 g L^{-1} and (D) 20 g L^{-1}.

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