Kinetic Analysis on Cell Growth and Biosynthesis of Poly (3-Hydroxybutyrate) (PHB) in Cupriavidus Necator H16

Maksudur Rahman Khan, D. M. Reddy Prasad, Hamidah Abdullah, and Abeed Fatima Mohidin Batcha

***Abstract*—Poly (3-hydroxybutyrate) (PHB) is a biodegradable polymer that can be synthesized through bacterial fermentation. In this study, *Cupriavidus necator* H16 was used to synthesize PHB by using jatropha oil as its sole carbon source. Experiments using 12.5 g/L of jatropha oil with the highest PHB accumulation of 8.6 g/L at 61.5hr was used to assess its kinetic pattern. Logistic and Leudeking-Piret model was used to evaluate the cell growth and PHB accumulation respectively and the theoretical values obtained corresponded well with the experimental data with slight deviation in cell growth and PHB accumulation at the end of stationary phase.**

***Index Terms*—*Cupriavidus necator*, jatropha oil, kinetic model, poly (3-hydroxybutyrate).**

I. Introduction

The current concerns over the increasing usage of non-biodegradable plastics and its impact to the nature have pushed researchers to develop bioplastics that are biodegradable and environmental-friendly. These bioplastics are mainly polyesters of polyhydroxyalkanoate (PHA) polymers which are produced by various microbes under nutrient-limiting conditions (e.g.: limitation of sodium and phosphorus) but with an excess of carbon source [1].

PHAs are 100% biodegradable and biocompatible polymers with properties such as thermoplastic, elastomer, insoluble in water and also non-toxic in nature [2]. These polyesters have characteristics similar to those of polyethene and polypropylene, and can therefore be used instead of conventional plastics. One particular example of PHA is Poly (3-hydroxybutyrate) (PHB) which is a homopolymer that contains monomers of 3-hydroxybutyrate. It has crystalline properties with a melting point of around 170^0C [3]. PHA has garnered great interest due to its biodegradability and biocompatibility with properties such as non-toxic, optically-active, and insoluble in water [2]. PHAs non-toxic property also makes it suitable for use in medical, pharmaceutical and also food industries [4].

Nevertheless, usages of bioplastics from PHA are still limited mainly because its production cost is still very high when compared to petroleum-based polyesters. Previous researches have proven that using substrate from pure glucose or sucrose can be rather expensive for large scale production. At USD 16/kg, the production cost of bioplastics from glucose would be 18 times higher than the conventional polypropylene plastics [6]. Since approximately 50% of PHA net production cost comes from the cost of its raw material [7], utilizing plant oil as the carbon source for PHA synthesis might reduce the PHA production cost and thus making large-scale PHA production a more feasible approach.

Meanwhile, researchers have also focussed on the idea of using industrial and municipal wastes for the synthesis of PHA. Previous researches have reported the use of various industrial wastes such as activated sludge, dairy waste, cheese whey, molasses and so on. In a research done by Chua *et al.* [8], it was shown that PHA accumulation was higher with slight alteration of the sludge by adding acetate into the municipal wastewater to give a PHA yield of up to 30% of the sludge's dry weight. Studies done by Rogers and Wu [9] suggested the use of enhanced biological phosphorus removal (EBPR) in activated sludge to give a yield of 50% PHA content under aerobic and anaerobic condition Cheese whey is another type of solid waste that can be used for PHA synthesis. As a by-product of dairy industry, it is considered as a pollutant due to its high content of [10]. In a study done by Pandian *et al.* [11] a yield of up to 11.32 g/L of PHB dry weight was obtained by using this dairy waste as their main substrate.

Utilizing industrial wastes as carbon source for PHA production might prove to be valuable since this approach has an added advantage of reducing sludge handling cost. However, the low yield of PHA obtained from this approach may not be feasible for large-scale PHA production. Previous researches such as the ones done by Park DH [12] gave promising results with up to 83% of PHB content with soybean oil as their carbon source. Meanwhile, López-Cuellar *et al*. [13] achieved 92% PHA content from fed-batch fermentation of *Cupriavidus necator sp.* by using canola oil as their carbon source. Similarly, Ng *et al.* [14] reported a total of 87% of PHB accumulation from the fermentation of *Cupriavidus necator sp.* by using jatropha oil as their main feedstock. Jatropha oil is known to have a high saturated fatty acid content [14] which will facilitate the synthesis of PHB. Apart from that, *C. necator* has been proven to accumulate PHB up to 80% of its cell dry weight [15].

Nonetheless, to ensure the efficiency of PHB production, it is essential to have a clear-cut mathematical model that could express the kinetic parameters such as cell dry weight and PHB concentration. A well-defined kinetic model could facilitate in problem-solving during large-scale fermentation

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process. In addition, it could also increase the production efficiency resulting in a better PHB yield. In the present study, poly-3-hydroxybutyrate (PHB) will be produced from the fermentation of *C. necator* using jatropha oil as its main carbon source. Although there were few reports on PHB production from jatropha oil, the kinetics involved during the bacterial fermentation still remain unclear. Therefore, this research focusses on establishing the kinetic study in predicting the metabolism of PHB production. We evaluated the effect of different variables (urea and oil concentration, agitation rate, etc.) on PHB production in our previous study (data not presented). From the results obtained, the kinetic model for cell growth and PHB production were fitted with our experimental data.

II. Materials And Methods

A. Microorganism and Culture Media

C. *necator* H16 (ATCC 17699) was used throughout this study. The mineral medium for the flask culture contained per litre: 1.5g KH_2PO_4, 9g Na_2HPO_4 .$12H_2O$, 0.2g MgSO.$7H_2O$, 1g urea and 1mL of trace element solution. Trace element solution contained per litre of HCl 0.1N solution: 0.3 g H_3BO_3, 0.2 g $CoCl_2$ $6H_2O$, 0.1 g $ZnSO_4$ $7H_2O$, 30mg $MnCl_2$ $4H_2O$, 30mg Na_2MoO_4 $2H_2O$, 20mg $NiCl_2$ $6H_2O$ and 10mg $CuSO_4$ $5H_2O$. Jatropha oil was autoclaved separately before it was added to the culture medium.

B. Growth Conditions

C. necator was pre-cultivated in nutrient-rich medium consisting of 2 g/L yeast extract, 10 g/L meat extract and 10 g/L peptone. 12 100mL shake flask containing 12.5g/L jatropha oil and 10mL mineral medium was inoculated with 400 µL of the pre-culture and incubated at 30^0C and 200rpm for 100 hours unless stated otherwise. The jatropha oil concentration used was based on previous study done by Ng *et al*. [14] on production of PHB from jatropha oil. To study the kinetics of PHB synthesis, one shake flask was taken at a certain time interval and the culture was centrifuged (8000 rpm, 10 min, 4^0C) in a pre-weighed centrifuge tube. Cell pellets were centrifuged twice after washing with hexane and distilled water respectively. The washed cell pellets were frozen at -20^0C for 24 h. These procedures were repeated for all the shake flasks and the frozen cell pellets were freeze dried and the cell dry weight (CDW) were obtained.

C. Analytical Procedure

The PHB concentration was determined with slight modifications from the method suggested by G. Braunegg *et al*. [16]. Approximately 10 to 20mg of lyophilized cells were subjected to methanolysis with 4mL of acidified methanol (10% (v/v) sulphuric acid) and 2mL of chloroform for 4 hours. 20mg of benzoic acid was also used as internal standard. The resulting 3-hydroxybutyric methyl esters (HBME) were analyzed by Gas Chromatography (GC) (6890N Series, Agilent Inc.) using HP-Innowax (30m × 0.25mm × 0.15µm). Initial column temperature was set to 80℃ with a temperature ramp of 5℃/min until a final temperature of 240℃ was reached. Helium was used as carrier gas at a flow rate of 20mL/min. Known amounts of pure PHB was treated similarly to obtain a calibration curve and the resulting PHB concentration was calculated based on the HBME peak areas obtained from the chromatograms.

D. Kinetic Modelling of Cell Growth and PHB Production

1) Cell growth rate

Logistic equation, a substrate-independent model was used to represent the rate of cell growth by determining the inhibition effect on cell growth. The logistic equation, originally proposed by Verhulst [17], can be described as follows:

$$\frac{dx}{dt} = \mu_m x(1-\frac{x}{x_m}) \tag{1}$$

where μ_m is the maximum specific growth rate (h^-) and x_m is the maximum cell concentration (g/L). The integrated form of eqn (1) gives the value of x as a function t.

$$x = x_0, t = t_0 \ ,$$

$$\text{Integrate to give } x = \frac{x_0 e^{\mu_m t}}{(1-\frac{x_0}{x_m})(1-e^{\mu_m t})} \quad t \le t_m \tag{2}$$

Rearrangement of equation (2) gives

$$\ln(\frac{x}{x_m - x}) = \mu_m t - \ln(\frac{x_m}{x_0} - 1) \tag{3}$$

2) PHB production rate

Since PHB synthesis in C. *necator* occurs during both exponential and stationary phase, Mulchandani et. al, [18] had suggested the use of Leudeking-Piret model to represent the PHB production rate associated with both the growth and non-growth stage. The model, originally proposed by Luedeking and Piret [19] can be described as follows:

$$\frac{dp}{dt} = \alpha \frac{dx}{dt} + \beta x \tag{4}$$

whereby α and β are the growth and non-growth associated constant, respectively. Substituting equation (1) and (2) into (4) and integrating will yield equation (5)

$$P = P_0 + \alpha x_0 \left[\frac{e^{\mu_m t}}{1-(x_0/x_m)(1-e^{\mu_m t})} - 1 \right] + \beta \frac{x_m}{\mu_m} \ln\left[1 - \frac{x_0}{x_m}(1-e^{\mu_m t}) \right] \tag{5}$$

Since PHB production starts mainly at exponential phase, the initial PHB concentration, P_0 was assumed to be negligible. Thus, Equation (5) can be simplified further as

$$P = \alpha A(t) + \beta B(t) \tag{6}$$

where $A(t) = x_0 \left[\frac{e^{\mu_m t}}{1-(x_0/x_m)(1-e^{\mu_m t})} - 1 \right]$ and

$B(t) = \frac{x_m}{\mu_m} \ln \left[1 - \frac{x_0}{x_m}(1-e^{\mu_m t}) \right]$ respectively.

At stationary phase, $\frac{dx}{dt} = 0$ and $x = x_m$. Therefore the value of β can be obtained from equation (4). The value of α can be obtained from the linear plot of $P = \beta B(t)$ against $A(t)$

III. Results And Discussion

In our previous research, the effect of different variables such as urea concentration, jatropha oil concentration and agitation rate on cell growth and PHB production were studied. The cell growth and PHB concentration obtained from bacterial fermentation of 12.5 g/L of jatropha oil were presented in Fig. 1. The growth curve has a classical pattern with an exponential phase up to 50hr followed by stationary phase that lasted until 65hr. Prolonged incubation time lead to decrease in both PHB concentration and CDW. The highest CDW of 11.6 g/L was obtained at 55hr followed by highest PHB concentration of 8.6 g/L at 61.5hr.

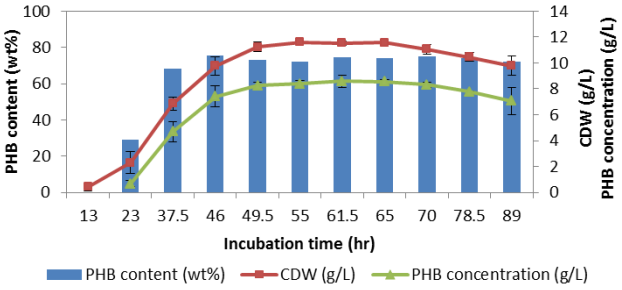

Fig. 1. Cell growth, PHB concentration and PHB content from bacterial fermentation of 12.5 g/L Jatropha oil with time. Urea concentration was fixed at 1 g/L. The cultivation was conducted in 100 mL shake flasks, and incubated at 30°C at 200 rpm for 90 hrs. All results were means of duplicate.

Based on the results obtained, the kinetic model for the experimental data was determined by using equations (1) to (6). The linear plot of equation (3) gives the values of μ_m and x_0 which are presented in Table I. Substituting these values along with x_m obtained from the experimental data into equation (2), yielded the theoretical CDW as depicted in Fig. 2. From the calculated constants, the values for A(t) and B(t) in equation (6) can be calculated to give the theoretical PHB concentration values (Fig. 2). The experimental data (Fig. 2) matches well with the kinetic model at log phase and early stationary phase. However, the experimental values deviate slightly towards the end of stationary phase because the logistic equation used does not portray the decrease in cell density that normally occurs at the end of stationary phase [20].

Similarly, the kinetic model for PHB concentration agrees well with the experimental data. The α value (0.6814 g/g) obtained was considerably higher than β (0.001768 g g^{-1} h^{-1}) which indicates that the PHB production mostly confirms to the growth associated kinetic pattern and only a small amount of PHB is produced during non-growth stage. The kinetic model appears to overestimate PHB production at the end of stationary phase. This is probably due to PHB degradation at the end of stationary phase that might have caused the PHB concentration to decline. Nevertheless, it should be noted that that the kinetic parameters may vary with change in fermentation condition such as agitation, pH and substrate concentration. Thus, the kinetic model for different experimental condition should be calculated individually.

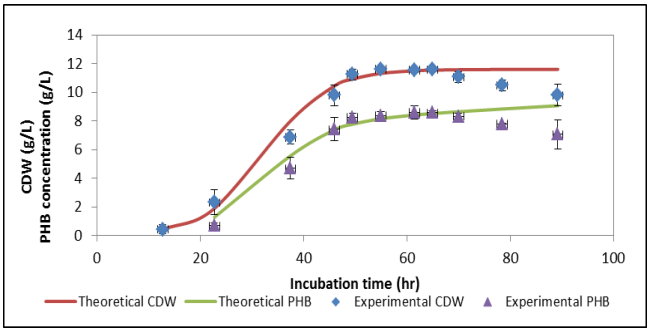

Fig. 2. Kinetic data for microbial cell dry weight and PHB concentration. Oil and urea concentration was fixed at 12.5 g/L and 1 g/L respectively. The cultivation was conducted in 100 mL shake flasks, and incubated at 30^0 C at 200 rpm for 90 hrs.

TABLE I: Summary of Kinetic Model Parameters

Constants	Values
Cell Growth Rate	
x_m (g/L)	11.59486
μ_m (h^{-1})	0.1651
x_0 (g/L)	0.0525
Product Formation Rate	
α (g/g)	0.6814
β (g g^{-1} h^{-1})	0.001768

IV. Conclusion

Jatropha oil has high potential to be used as carbon source in bacterial biopolymer production. The cell growth and PHB concentration from experimental data agrees well with the logistic and Leudeking-Piret model. The PHB accumulation mostly adhered to growth-associated kinetic pattern with insignificant amount of PHB produced during the non-growth stage.

Appendix

P	Product concentration (g/L)
P_0	Initial product concentration (g/L)
t	Time (h)
t_m	Time when maximum cell concentration reached (h)
x	Cell concentration (g/L)
x_0	Initial cell concentration (g/L)
x_m	Maximum cell concentration (g/L)
α	Growth associated constant (g/g)
β	Non-growth associated constant (g g^{-1} h^{-1})
μ_{max}	Maximum specific cell growth rate (h^{-1})

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