Microbial production of poly (3-hydroxybutyrate) (PHB) from rubber seed oil using *Cupriavidus necator* H16

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**Abstract.** Rubber seed oil is inedible plant oil that is a potential replacement for the traditional organic building blocks used in PHB biosynthesis. Herein we describe the biosynthesis of PHB by *Cupriavidus necator* H16 grown in media with rubber seed oil and urea serving as carbon and nitrogen sources, respectively. The method was optimized over a temperature range of 30-50 °C for batch culture studies. A maximized PHB concentration of 15.4 g/L, and dry well weight of 19.2 g/L was obtained using 20 g/L of rubber seed oil at 30°C. Experimental results were used to generate a logistic curve, and the Luedeking-Piret model was implemented to predict the specific growth rate of *C. necator* at 0.1898 1/h. The estimated rate constants were in good agreement with experimental values. This study demonstrated that rubber seed oil is a viable alternative to conventional carbon sources for the production of PHB using *C.necator* H16.

**List of Symbols**

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| Symbol | Description                   |
|--------|-------------------------------|
| P      | Product concentration (g/L)   |
| P₀     | Initial product concentration (g/L) |
| t      | Time (h)                      |
| tₘₐₓ  | Time when maximum cell concentration is reached(h) |
| x      | Cell concentration (g/L)       |
| x₀     | Initial cell concentration (g/L) |
| xₘₐₓ  | Maximum cell concentration (g/L) |

**Greek letters**

| Symbol | Description                   |
|--------|-------------------------------|
| α      | Growth associated constant (g/g) |
| β      | Non-growth associated constant (g/g h) |
| μₘₐₓ  | Maximum specific cell growth rate (1/h) |

**1. Introduction**

Throughout the years, the benefits of commonplace plastics have been demonstrated in the economy, environment, and society. Approximately 311 million tonnes of plastic were made in 2010, where roughly 23 percent of these products used in the European Union [1]. Around half of the manufactured...
plastic is used to create disposable products such as agricultural films, packaging materials and consumable goods [2]. The generation of 600 to 1300 million tonnes of CO2 is avoided by using plastics through replacement of less efficient materials, fuel savings in transport, application in wind power rotors, contribution to insulation, photovoltaic cells and prevention of food losses [3]. Unfortunately, their applicability has resulted in environmental damage. The production of large amounts of plastic waste has caused environmental problems, public health crises, and economic collapses. Moreover, as the significant unprocessed material used in plastic production, petroleum is on the brink of depletion, as sources are barely sufficient for supplying global fuel demands. Additional petroleum consumption via plastic production only exacerbates this issue, so that the rising costs of plastic manufacture will eventually translate to higher prices for plastic products. Plastic production consumes about 8 percent of the oil produced in the world each year; specifically, 4 percent is used as raw materials while 3 to 4 percent is consumed during manufacture [1]. While current statistics for oil consumption in plastic production do not exceed 10 percent, the demand for plastic for the next few years shows an upward trend, especially in the vital medial, pharmaceutical, and automotive industries; as such, it is likely that petroleum consumption will only increase to meet the rising demand for plastics around the world.

Many strategies have been implemented to address the adverse effects of conventional plastics. One of the most widely employed methods is recycling, but it is not economically effective in the long term due to the difficulty and added costs of separating and sorting the different types of plastics; furthermore, the presence of additives in plastics limits the applications of these recycled materials due to possible health and environmental concerns [4]. Therefore, attention should be focussed on decreasing the lifetime and increasing the biodegradability of conventional plastics so that they can easily degrade after a short duration. The manufacture and commercialization of eco-friendly bioplastics can avoid worsening the negative consequences of pollution caused by conventional plastics [5].

Therefore, the focus of this study is on completely biodegradable plastics which include common biopolymers such as polylactides (PLAs), aliphatic polyesters, polyhydroxyalkanoates (PHAs), polysaccharides, copolymers and a combination of the above [6]. PHA is a non-toxic, water-insoluble, biocompatible and elastomeric thermoplastic that is entirely biodegradable. Its chemical properties are similar to those of polyethylene and polypropylene so that it is a possible alternative to conventional plastics [7]. Poly(3-hydroxybutyrate) (PHB) is the most well-characterized PHA.

However, the major obstacle to the replacement of conventional plastics with PHA is the significantly higher market price of the latter [8]. PHA prices are dependent on the costs of the use of high-purity substrates like propionate and glucose, (P(HB-HV) manufacture and the downstream process for polymer extraction [9]. Many issues need to be resolved to make PHA production economically viable. For example, cost-effective bioplastic fabrication is dependent on the use of highly productive microorganisms and cheap raw materials. Specifically, the type of substrate greatly influences the yield, composition and chemical properties of the biosynthesized PHA; besides, 40 to 50% of total production costs are spent on raw materials [10].

The selection of a carbon source is a major goal for optimizing PHB biosynthesis. As previously mentioned, the nature of the carbon source affects the yield, content and chemical properties of the PHB polymer. Furthermore, up to 80% of final PHB production costs are spent on the carbon substrate, so that economical production costs can be achieved by using an affordable source of carbon. Forest seed oils such as soybean, rubber seed, olive, coconut and sunflower oil are food-grade oils that are excellent carbon sources for PHA production, although there may be ethical concerns that repurposing food products for bioplastic manufacture is wasteful. However, these concerns do not apply to rubber seed oil, and it is a potential substitute for conventional feedstock for high-yield production of PHA.

Unlike industrial waste oils, the oils from renewable sources are very viable candidates for carbon substrates used in PHA production. For instance, soybean oil was used as the carbon source to produce PHA with yields as high as 83% [11]. Meanwhile, fed-batch fermentation of C. necator using canola oil feedstock yielded a PHA content of 92% [12] similarly, a total PHB output of 87% was reported using jatropha oil [13]. The wild strain C. necator H16 has been found to accumulate PHB to as much as 80% of its cell dry weight [14]. Furthermore, a significant fraction of overall production costs are incurred by the PHB recovery process, which can be significantly mitigated or avoided by increasing the PHB
yield [15]. Inedible oils like rubber seed oil and jatropha oil are preferred over edible oils since usage of the former in PHB production would not exacerbate the global food crisis [16].

Herein, we explore the effects of procedural parameters (i.e., urea and oil concentration and temperature) on PHB biosynthesis using C. necator H16 with rubber seed oil as the carbon source. The fermentation conditions were optimized to enhance the PHB yield. Additionally, a biokinetic study was also performed to determine microorganism growth and metabolism rates during PHB biosynthesis.

2. Methodology

2.1 Microorganism and culture media

Cupriavidus necator H16 (ATCC 17699) was cultured in flasks for PHB biosynthesis. The mineral medium consisted of 1 mL of trace element solution, 1.5 g of KH₂PO₄, 9 g of Na₃HPO₄·12H₂O, 0.2 g of MgSO₄·7H₂O and 1 g of urea per liter. The trace element solution was prepared in 0.1 N of HCl and comprised of 0.3 g of H₃BO₃, 0.2 g of CoCl₂·6H₂O, 0.1 g of ZnSO₄·7H₂O, 30 mg of MnCl₂·4H₂O, 30 mg of Na₂MoO₄·2H₂O, 20 mg of NiCl₂·6H₂O and 10 mg of CuSO₄·5H₂O per liter. Rubber seed oil was autoclaved separately before its addition to the culture medium [17].

2.2 Growth conditions

C. necator was pre-cultured in a nutrient-rich medium consisting of 10 g/L of meat extract, 10 g/L of peptone and 2 g/L of yeast extract. Ten 100 mL shake flasks containing 15 g/L of rubber seed oil and 10 mL of mineral medium were inoculated with 0.4 mL of the pre-culture and incubated for 100 h at 30°C and 200 rpm unless stated otherwise. The concentration of rubber seed oil used for PHB production was similar to that for jatropha oil in a previous study [13], since both are non-edible plant oils. One shake flask was taken at a certain time interval to study the kinetics of PHB biosynthesis, and the culture was placed in a pre-weighed centrifuge tube and centrifuged for 10 min at 4 °C and 8000 rpm. Cell pellets were centrifuged twice after washing with hexane and distilled water, and the washed pellets were then frozen at -20°C for 24 h. These procedures were repeated for all the shake flasks, after which the frozen pellets were freeze-dried, and their cell dry weights (CDWs) obtained [17].

2.3 Analytical procedure

PHB concentrations were determined using a previously reported method [18]. Approximately 10-20 mg of lyophilized cells were subjected to methanolysis for 4 h in 4 mL of acidified methanol (10 % sulphuric acid, v/v) and 2 mL of chloroform. An internal standard of 20 mg of benzoic acid was also added. The resulting 3-hydroxybutyric methyl esters (HBME) were analyzed by gas chromatography (GC, 6890N Series, Agilent Technologies) using an HP-INNO wax column (30 m x 0.25 mm x 0.15 μm). The initial column temperature was set at 80°C and ramped to a final temperature of 240°C with a gradient of 5°C/min. Helium was used as the carrier gas with a flow rate of 20 mL/min. Known amounts of pure PHB were analyzed to generate a calibration curve so that unknown PHB concentrations were calculated based on the HBME peak areas obtained from the chromatograms.

3. Kinetic modeling of cell growth and PHB production

3.1 Cell growth rate

To determine the growth rate of C. necator, a substrate-independent model was used to evaluate the effects of inhibition on bacterial growth. The model utilizes the below logistic equation that was first proposed Verhulst P [19]:

\[ \frac{dP}{dt} = rP \left( 1 - \frac{P}{K} \right) \]  

(1)

where \( P \) and \( K \) represent the population size and carrying capacity, respectively, and the growth rate is defined by the constant \( r \). The above equation was modified for C. necator growth into the below expression:

\[ \frac{dx}{dt} = \mu_m x \left( 1 - \frac{x}{x_m} \right) \]  

(2)
where $\mu_m$ is the maximum specific growth rate (1/h), and $x_m$ is the maximum cell concentration (g/L). Integrating Equation 2 gives the value of $x$ as a function of $t$, so that integrating $x_0$ to $x$ and $t_0$ to $t$ gives the below equation:

$$x = \frac{x_0 e^{\mu_m t}}{1 - (\frac{x_0}{x_m})(1 - e^{-\mu_m t})}, \quad t \leq t_m$$  \hfill (3)

Rearrangement of Equation 3 results in the following formula:

$$\ln \left( \frac{x}{x_m - x} \right) = \mu_m t - \ln \left( \frac{x_m}{x_0} - 1 \right)$$  \hfill (4)

### 3.2 PHB production rate

Since PHB biosynthesis in *C. necator* occurs during both exponential and stationary phases, the use of the Luedeking–Piret model was proposed to determine the PHB production rate in both stages [20]. The model, proposed initially by Luedeking and Piret in 1959 [21], is as follows:

$$\frac{dP}{dt} = \alpha \frac{dx}{dt} + \beta x$$  \hfill (5)

where $\alpha$ and $\beta$ are constants associated with the exponential and stationary stage, respectively. Substituting Equations 2 and 3 into Equation 5 follows by integration yields Equation 6:

$$P = P_0 + ax_0 \left[ \frac{x_0 e^{\mu_m t}}{1 - (\frac{x_0}{x_m})(1 - e^{-\mu_m t})} - 1 \right] + \beta x_m \ln \left[ 1 - \left( \frac{x_0}{x_m} \right) \left( 1 - e^{-\mu_m t} \right) \right]$$  \hfill (6)

Because the majority of PHB is produced during the exponential phase, the initial PHB concentration $P_0$ was assumed to be negligible. Thus, Equation 6 can be further simplified to generate the expression:

$$P = \alpha A(t) + \beta B(t)$$  \hfill (7)

where

$$A(t) = x_0 \left[ \frac{x_0 e^{\mu_m t}}{1 - (\frac{x_0}{x_m})(1 - e^{-\mu_m t})} - 1 \right]$$  \hfill (8)

and

$$B(t) = x_m \ln \left[ 1 - \left( \frac{x_0}{x_m} \right) \left( 1 - e^{-\mu_m t} \right) \right]$$  \hfill (9)

During the stationary phase, $dx/dt = 0$ and $x = x_m$. Therefore, $\beta$ can be determined from Equation 5, while $\alpha$ can be calculated from the linear plot of $[P - \beta B(t)]$ against $A(t)$.

### 4. Results and discussion

#### 4.1 Effect of urea concentration on growth

The urea concentration increased from 0.5 g/L to 1.5 g/L the CDW of *C. necator* was not affected much. The small incremental value is 0.001 (Figure 1). At a urea concentration of 1.0 g/L, the maximum CDW was 13.3 g/L, and the concentration of PHB produced was 9.2 g/L. We observed that the CDW slowly decreased at urea concentrations above 1.0 g/L. A rubber seed oil concentration of 15 g/L was used throughout the study. All cultures were grown in 100 mL shake flasks and incubated for 90 h at 30°C and 200 rpm. Analyses were performed in duplicate.

Results show that urea is an effective source of nitrogen, since slightly elevating the amount of urea significantly increased bacterial CDWs. The nitrogen limit at optimized urea levels is sufficient for both bacterial growth and PHB biosynthesis, during which nitrogen is consumed and stored by cells to accommodate the accumulation of bioplastic. Conversely, the nitrogen limit at low urea concentrations is insufficient for bacterial growth and PHB accumulation; instead of amassing PHB, cells consume the existing PHB, resulting in low yields. Therefore, urea concentration is an inhibiting factor for cell growth. Cell growth was saturated at high urea levels due to an excess of nitrogen, precisely due to an increase in the pH of the culture environment. Sudden changes in pH can induce cell lysis and increase PHB accumulation; adjustment of the carbon to nitrogen ratio can be made to prevent this phenomenon.

The effectiveness of urea as a nitrogen source has been reported. By using urea, we obtained a CDW and accumulated PHB concentration of 13.3 g/L and 9.6 g/L, respectively (Figure
These figures demonstrate that urea is the most effective nitrogen source as opposed to ammonium sulphate, ammonium nitrate and ammonium chloride [22].

Figure 1. Influence of urea on CDW of *C. necator* and production of PHB.

4.2 Effect of rubber seed oil concentration on growth

Figure 2 shows the effects of different rubber seed oil concentrations on CDW and PHB, where cell growth increased with oil concentrations. Based on the figure, the CDW significantly increased when the oil concentration was increased from 12 to 16 g/L, but stayed constant above 16 g/L. A maximum CDW of 19.2 g/L was observed at an oil concentration of 20 g/L.

Using rubber seed oil as the carbon feedstock resulted in a satisfying PHB yield of 15.4 g/L. The unique free fatty acid content in rubber seed oil enables the production of more PHB than when employing conventional substrates. Since rubber seed oil is the carbon source, its concentration is positively
correlated to the CDW, and cell growth is inhibited by the levels of carbon substrates in the system. However, there is a threshold after which bacteria start to convert PHB back into energy and carbon substrate. While PHB amassment is limited in cultures with low cell counts, rubber seed oil concentrations up to 20 g/L exhibited a constant CDW, so that high rubber seed oil levels did not affect bacterial proliferation. However, the CDW is positively correlated with oil concentrations below 16 g/L; the oil is comprised of free fatty acids such as palmitic, linoleic and oleic acid that are conducive to optimal bacterial growth.

Rubber seed oil has been implemented to fabricate various PHAs with short-chain monomers that are 3-5 carbons long, medium-chain monomers that are 6-14 carbons long or a combination of both. Regardless of monomer chain length, free fatty acids in the oil are hydrolyzed by extracellular lipase and taken up by bacteria before proliferation and PHA biosynthesis [23, 24]. It is essential to investigate free fatty acid mixtures because they contain carbon and energy sources that are necessary for bacterial growth and PHB production. It has been reported that raising the medium concentrations of rubber seed oil will not improve its uptake by cells [25]. Furthermore, studies showed that rubber seed oil should be introduced to cells via controlled feeding to circumvent the toxic effects of free fatty acids on bacterial growth. During cell growth with jatropha oil, C. necator cells were found to utilize palmitic and oleic acids more effectively than linoleic acid to produce 4.4 g/L of total P(3HB) [13].

**Figure 2.** Influence of rubber seed oil concentration on CDW of C. necator and production of PHB

4.3 Effect of temperature on growth

Since C. necator can grow over a wide temperature range, the effects of temperature on bacterial growth and PHA production were studied from 30 to 60°C in increments of 10°C. Results indicated that a temperature of 30°C was optimal for C. necator growth and PHA production, where proliferation and bioplastic production was lowest at 30 and 60°C (Figure 3), which is in good agreement with previous C. necator studies on PHA production [26].
4.4 Kinetic model prediction

A kinetic model for microbial fermentation using 15 g/L of rubber seed oil and 1 g/L of urea was determined using Equations 2-7. The linear plot in Equation 4 was used to calculate the values of $\mu_m$ and $x_0$ which are seen in Table 1. Substituting these values with $x_m$ obtained from the experimental data into Equation 3 yielded a theoretical CDW as shown in Figure 4. The values for $A(t)$ and $B(t)$ in Equation 7 can be determined using these constants to yield theoretical PHB concentrations (Figure 4). As illustrated in Figure 4, the experimental data is in agreement with the kinetic model during the exponential and early stationary phases, but deviate slightly near the end of the stationary phase because the logistic equation did not show the characteristic decrease at that stage [27]. Similarly, the kinetic model for PHB concentration agrees with experimental data. The calculated $\alpha$ value (0.7836 g/g) was considerably higher than that of $\beta$ (0.002033 g/g h), indicating that PHB production is consistent with the growth-associated kinetic pattern, with low production rates during non-growth stages. Towards the end of the stationary phase, the kinetic model appears to overestimate the amount of biosynthesized PHB, which may be due to PHB degradation near the death phase that was reflected in our experimental data. As kinetic parameters are influenced by changes in fermentation conditions such as pH, agitation and substrate concentration, the kinetic model should be individually calculated for different experimental conditions.

![Kinetic data for microbial CDW and PHB concentrations.](image)

**Table 1.** Summary of kinetic model parameters for microbial cell growth and PHB formation rate

| S.No | Constant     | Value         |
|------|--------------|---------------|
| 1    | $x_m$ (g/h)  | 13.3340       |
| 2    | $\mu_m$ (1/h)| 0.1898        |
| 3    | $x_0$ (g/h)  | 0.0603        |
| 4    | $\alpha$ (g/g)| 0.7836       |
| 5    | $\beta$ (g/g h)| 0.002033    |
5. Conclusions

In this study, rubber seed oil proved to be a viable and effective carbon source that was utilized to produce high yields of PHB. Experimental results indicated that the final biomass exceeded 1 g of biomass per 1 g of rubber seed oil. Oil and urea concentrations were optimized at 15 to 20 g/L and 1 to 1.5 g/L, respectively. The highest CDW was amassed after an incubation period of 48 to 60 h. The total biomass of C. necator grown in the presence of 1 g/L urea was larger than C. necator grown without urea. The maximum specific cell growth rate $\mu_m$ is 0.1898 1/h is reasonable, and the mathematical model for bacterial proliferation and PHB accumulation was well-fitted, albeit with a slight deviation at the end of the death phase.

6. References

[1] Mudgal, S., Lyons, L., Bain, J. 2010 Plastic waste in the environment – final report for European Commission DG Environment. Bio Intelligence Service. Retrieved February 11, 2012, http://www.ec.europa.eu/environment/waste/studies/pdf/plastics.pdf

[2] Hopewell, J., Dvorak, R., Kosior, E., Plastics recycling: challenges and opportunities. Philosophical. Trans R Soc Lond B, 364 (2009) 2115-212.

[3] Plastics Europe (2010) Plastics – the Facts 2010, An analysis of European Plastics production, demand and recovery for 2009. http://www.plasticeurope.org/document/plastics---the-facts-2010.aspx?FoilID=2

[4] Flechter, A., PHA as natural, biodegradable polyesters. In Plastics from bacteria and for bacteria, (1993) 77-93. Springer Verlag, New York, USA.

[5] Song, S.S., Hein, S., Steinbuchel, A., Production of poly (4-hydroxybutyric acid) by fed-batch cultures of recombinant strains of Escherichia coli. Biotechnol.Lett, 21(1999) 193–197.

[6] Reddy, C., Ghai, R. Rashmi, & Kalia, V., Poly-hydroxyalkanoates: an overview. Bioresource Technol 87(2003) 137-146.

[7] Kulkarni S.O., P.P. Kanekar, S.S. Nilegaonkar, S.S. Sarnaik, J.P. Jog, Production and characterization of a biodegradable poly (hydroxybutrate-co-hydroxyvalerate) (PHB-co-PHV) copolymer by moderately haloalkalitolerant Halomonas campisalis MCM B-1027 isolated from Lonar Lake, India, Bioresource Technol 101 (2010) 9765–9771

[8] Bibly, G.D. (2002) Degradable plastics. Retrieved January 12, 2012, http://www.icma.com/info/polymers.htm.

[9] Lee, S.Y., Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends Biotechnol, 14 (1996) 431-438.

[10] Meesters, K. H. P., Production of poly (3 hydroxyalkanoates) from waste streams. (1998) Report of Technical University of Delft, Delft, Netherlands.

[11] Park DH, Kim BS, Production of poly (3-hydroxybutyrate) and poly (3-hydroxybutyrate-co-4-hydroxybutyrate) by Ralstonia eutropha from soybean oil. New Biotechnol 28(6) (2011) 719-724.

[12] Lo´pez-Cuellar M, Alba-Flores J, Rodr´iguez JG, Pe´rez-Guevara F, Production of polyhydroxyalkanoates (PHAs) with canola oil as carbon source. Int J Biol Macromol 48 (2010) 74–80.

[13] Ng KS, Ooi WY, Goh LK, Shenbagarathai R, Sudesh K, Evaluation of jatropha oil to produce poly(3-hydroxybutyrate) by Cupriavidus necator H16. Polym Degrad Stab 95 (2010) 1365–1369.

[14] Reinecke F, Steinb¨uchel A, Ralstonia eutropha strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers. J Mol Microbiol Biotechnol 16 (2009) 91–108.

[15] Choi JI, Lee SY, Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation. Bioprocess Eng 17 (1997) 335–342.

[16] Nakamura, S., Kunioka, M., Doi, Y., Biosynthesis and characterization of bacterial poly (3-hydroxybutyrate-co-3-hydroxypropionate). J. Macromol.Sci.Chem. 28 (1991) 15–24.
[17] Khanna S, Srivastava A, Recent advances in microbial polyhydroxyalkanoates. *Process Biochem* 40 (2005) 607–619

[18] Braunegg G, Sonnleimer B, Lafferty R, A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. *Eur J Appl Microbiol Biotechnol* 6(1978) 29–37.

[19] Verhulst P, Notice sur la loi que la population poursuit dans son accroissement. Correspondance mathe matique et physique 10(1838) 113–121.

[20] Mulchandani A, Luong JH, Groom C, Substrate inhibition kinetics for microbial growth and synthesis of poly-β-hydroxybutyric acid by *Alcaligenes eutrophus* ATCC 17697. *Appl Microbiol Biotechnol* 30 (1989) 11–17

[21] Luedeking R, Piret EL, A kinetic study of the lactic acid fermentation. Batch process at controlled pH. *J Biochem Microbiol Technol Eng* 1(1959) 393–412

[22] Srivastava, A. K., Khanna, S. Statistical media optimization studies for growth and PHB production by *Ralstonia eutropha*. *Process Biochem*, 40 (2005) 2173-2182.

[23] Sudek K, Bhubalan K, Chuah J-A, Kek Y-K, Kamilah H, Sridewi N, Lee Y-F, Synthesis of polyhydroxyalkanoate from palm oil and some new applications. *Appl Microbiol Biot* 89(2011) 1373–1386.

[24] Silk NJ, Denby S, Lewis G, Kuiper M, Hatton D, Field R, Baganz F, Lye GJ, Fed-batch operation of an industrial cell culture process in shaken microwells. Biotechnol Lett 32(2010) 73–78.

[25] Annuar, M.S.M., Tan, I.K.P., Ibrahim, S. and Ramachandran, K.B., Production of medium-chain-length poly(3-hydroxyalkanoates) from crude fatty acids mixture by *pseudo monas putida*, *Trans IChemE*, Part C, *Food and Bioproducts Processing*, 85(C2) (2007) 104–119.

[26] Der-Shyan Sheu, Wen-Ming Chen, Yung-Wei Lai, and Rey-Chang Chang, Mutations Derived from the Thermophilic Polyhydroxyalkanoate Synthase PhaC Enhance the Thermostability and Activity of PhaC from *Cupriavidus necator* H16, *J. Bacteriol*. 194 (10) (2012) 2620-2629.

[27] Wachenheim DE, Patterson JA, Ladisch MR, Analysis of the logistic function model: derivation and applications specific to batch cultured microorganisms. *Bioresour Technol* 86 (2003) 157–164.