

Full Length Research Paper

Kinetic model for polyhydroxybutyrate (PHB) production by *Hydrogenophaga pseudoflava* and verification of growth conditions

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Accepted 29 March, 2010

A kinetic model that describes microbial growth, biopolymer production and substrate consumption is used to predict the performance of batch fermentation of *Hydrogenophaga pseudoflava*. *H. pseudoflava* DSMZ 1034 is useful in synthesizing polyhydroxyalkanoates (PHAs). The experimental data was also fitted with the logistic equation that can provide adequate description for PHA synthesized by *H. pseudoflava*. The Lineweaver-Burk plot defined biokinetic coefficients which were described by a simplified Monod's rate model. The specific growth rates, μ_{max} and the Monod constants, K_s , for various substrates such as glucose, fructose were 0.36, 0.24, h^{-1} and 106, 80 g/l, respectively. A good agreement was found between the experimental and the predicted values, which indicated that the model with differential equations would describe fermentation process for the PHA formation.

Key words: Kinetic model, polyhydroxyalkanoates, *Hydrogenophaga pseudoflava*, logistic model, Monod, biopolymer.

INTRODUCTION

Polyhydroxybutyrate (PHB) is a biopolymer that can be used as a biodegradable thermoplastic material for waste management strategies and biocompatibility in medical devices (Gouda et al, 2001). The viability of microbial large scale production of PHB is dependent on the development of a low cost process that produces biodegradable

plastics with properties similar or superior to petrochemical plastics (Doi and Steinbüchel, 2001; Sims, 2003; Apostolis et al., 2006). The commercial production of PHB has been using relatively cheap substrates such as methanol, beet molasses, ethanol, starch and whey (Sharifzadeh et al., 2009 a, b; Kim, 2000; Ghaly et al., 2003), cane molasses as a sole carbon source (Gouda et al., 2001), wheat hydrolysate and fungal extract (Apostolis et al., 2006) or soy cake (Fabiane et al., 2007). Various nitrogen-rich media, such as casein hydrolysate, yeast extract, typtone, corn steep liquor and collagen hydrolysate (Khanna and Srivastava, 2005), have been used in PHB bio-conversions using either *Hydrogenophaga pseudoflava* recombinant *Esche-richia coli* strains. However, unrefined carbon sources such as corn syrup, cane molasses, beet molasses, or malt extract, also support PHB formation, obtaining yields of PHB comparable to, even better than the refined sugars. Polyhydroxyalkanoates (PHA) production by wild type bacterial strains occurs under nutrient depletion conditions. In the PHA production phase, the cell growth is limited owing to the depletion of essential nutrients such as nitrogen,

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Nomenclature: **S**, Substrate concentration (g/ l); **S₀**, initial substrate concentration (g/ l); **t**, ferment time (h); **X**, cell concentration (g/ l); **X_m**, maximum cell concentration (g/ l); **X₀**, initial cell concentration (g/ l); **K_s**, monod constant (g/l); **Y_{X/S}**, yield factor for cells on carbon substrate (g cells/ (g substrate)); **Y_{P/S}**, yield factor for product on carbon substrate (g polyhydroxybutyrate / (g substrate)); **μ_m**, maximum specific growth rate (1/h); **μ**, specific growth rate (1/h); **PHAs**, polyhydroxyalkanoates; **PHB**, polyhydroxybutyrate; **CDW**, cell dry weight; **GC**, gas chromatography; **FID**, flame ionization detector.

phosphorus, magnesium, among others.

This depletion in the presence of excess carbon source triggers the metabolic shift from growth to PHA production phase. *H. pseudoflava* has been used for optimal production of PHB, a homopolymer that is accumulated under nitrogen limitation (Morinaga et al., 1978). *Azotobacter beijerinckii* produces PHA under oxygen limitation compared to nitrogen or from fermentation of different carbon sources (Valappil et al., 2007; Kumar et al., 2007; Labuzek and Radecka, 2001). PHA production is a complex process wherein the final quality and quantity of the product yield depends on the strain, metabolic pathway involved, fermentation parameters, PHA production phase (stationary, throughout growth), carbon source and nutrient depletion condition required for PHA synthesis. In order to optimize PHA fermentation process, the kinetics of PHA production in *H. pseudoflava* has been examined in the present work. Useful kinetic model for biopolymer synthesis could include balances on cell mass, product concentration, substrate utilization and a single limiting substrate (Divyashree et al., 2009). One of the very important practical applications of this model is the evaluation of the product formation kinetics. Mathematical models facilitate data analysis and provide a strategy for solving problems encountered in fermentations. Information on fermentation process kinetics is potentially valuable for the improvement of batch process performance. Finally, the product yields and substrate conversions are criteria with the main attention toward productivity. The Monod kinetic model used for PHAs production is described by the following equation:

$$\mu = \frac{\mu_m S}{K_S + S} \quad (1)$$

Where, μ is the specific growth rate (h^{-1}), S is substrate concentration (g/l) and the terms K_S and μ_m are defined as Monod constant (g/l) and maximum specific growth rate, respectively.

The most active part of the cell growth curve is the exponential (log) phase which is used for the determination of kinetic parameters. The log phase is a period of balanced growth, in which all components of a cell grow at the same rate (Divyashree et al., 2009). Malthus model was also used for the cell growth behavior. The derivatives for biomass generation with respect to time, is related to specific growth rate which is defined as follows (Divyashree et al., 2009):

$$\mu = \frac{1}{X} \frac{dX}{dt} \quad (2)$$

Where, X is the cell mass concentration (g/l) and t is time (h). By separation of variables and integrating Equation 2 yields:

$$\ln \frac{X}{X_0} = \mu t \quad (3)$$

Where, X is biomass concentration with respect to time and X_0 is the initial biomass concentration.

The substrate and product inhibitory effect on cell growth has been investigated in the literature (Najaf pour, 2007). The cell growth rate was evaluated based on growth kinetics. Logistic equation was a suitable kinetic model for prediction of growth curve. The specific growth rate is predicted by Logistic model presented by Equation 4.

$$\mu = \mu_m \left(1 - \frac{X}{X_m}\right) \quad (4)$$

Where, X is the maximum cell dry weight concentration (g/l). By substitution of Equation 4 into Equation 2 and performing integration, the following equation for the cell concentration was obtained (Khanna and Srivastava, 2005):

$$X = \frac{X_0 \exp(\mu_m t)}{1 - \left(\frac{X_0}{X_m}\right)(1 - \exp(\mu_m t))} \quad (5)$$

The above equation was used to predict the cell growth in batch experiments. In this research, inoculation volumes were kept constant for batch experiments. The logistic model was a good approximation of the growth curve. Matlab (V 7.4) computer software was used to define logistic growth kinetic parameters.

The purpose of the present research was to investigate the effect of various carbon sources such as glucose, fructose on biopolymer production. Kinetic parameters for the cell growth were determined.

MATERIALS AND METHODS

Microorganism

The microorganism used in the present study was *H. pseudoflava* DSMZ 1034 (Deutsche Sammlung von Mikroorganismen und Zellkulturen) for culture propagation. The stock culture was stored and maintained on Luria agar slants at 4°C. The organism was sub-cultured every 15 days to maintain its viability.

Media

Glucose and fructose were used as standard substrates. Seed culture and fermentation medium were the same with the following contents: 3.57 g Na_2HPO_4 , 1.35 g $(NH_4)_2SO_4$ and 0.5 mL trace element solution and 1 L distilled water. Different amounts of glucose and fructose were used in fermentation medium but 10 g of it was used in seed culture. Trace element solution contents were 2 g $MgSO_4$, 1 g $FeSO_4$, 0.8 g $MnSO_4$, 4 g K_2SO_4 , 0.2 g H_3BO_3 and 0.4 g $CuSO_4$ in 100 mL distilled water. The media were autoclaved at 121°C for 20 min. Fructose and glucose with specific amount in every test was

Table 1. Kinetic parameters for the PHB production with various carbon sources.

Carbon source	Glucose	Fructose
Sugar consumed (%)	56	59
Maximum biomass yield (g/g)	0.53	0.69
Maximum product yield (g/g)	0.25	0.17
Monod constant K_s (g/l)	106	80
Monod μ_{max} (h^{-1})	0.36	0.24
Logistic μ_{max} (h^{-1})	0.18	0.14

autoclaved in a separate container. The mineral and sugars solutions were mixed prior to inoculation. The media was adjusted to pH 7.0 by 1 M NaOH.

Experimental conditions

Stock culture in slants of *H. pseudoflava* was incubated at 30°C for 24 h. The resultant cultures were transferred into 500 ml flasks containing 100 ml medium. The flasks were incubated at 30°C and agitated at 250 rpm for 10, 15, 20 and 30 h, to investigate the effect of the seed age on biopolymer production. The inoculum size was 5% of the medium. Then, the incubation temperatures were set at the following conditions: temperature at 30°C, agitation rate of 250 rpm according to (Sharifzadeh Baei et al., 2009). For the above experimental conditions, the cell dry weight (CDW) and PHAs accumulation inside the cells were investigated.

Cell dry weight

The cell concentration in the cultured media was determined by the cell optical density at wavelength of 620 nm using spectrophotometer (UNICO2100, USA) with distilled water for suitable dilution. The cell dry weight was also measured based on standard calibration curve for cell optical density absorbance as a function of cell dry weight for the pure cultured of *H. pseudoflava*

Carbohydrate concentration

The supernatant obtained from centrifuged solution was used for residual nutrient analysis including total carbohydrates according to the method developed by reduced sugar analysis using 3,5-dinitrosalicilic acid (DNS) method (Miller, 1959).

Biopolymer analysis

For PHB quantification, 5 ml of culture broth was centrifuged at 3600 rpm for 20 min. A 2 ml solution of chloroform and 2 ml of acidified methanol (3% sulfuric acid) were added to the cell pellet in a vial with Teflon screw cap and heated at 100°C for 3.5 h. The developed extraction method was based on experimental method developed by Braunegg et al. (1987).

Gas chromatography (GC) was performed using a gas chromatograph (Philips PU4400, US) equipped with flame ionization detector (FID) and data acquisition system with computer software (Clarity 4.2, Data Apex, Czech Republic). The GC was used for the methyl-3-hydroxybutyrate (3 HB) analysis. The GC was equipped with capillary column (BP20 SGE, Australia), 0.33 mm internal diameter and 25 m length. The column temperature was initially maintained

at 80°C for 4 min, followed by the temperature programming at a rate of 8°C/min till it reached 160°C, maintained for 3 min and then at a rate of 30°C/min increased to 200°C. The detector and injector temperatures were 280 and 250°C, respectively. The carrier gas used were helium with a flow rate of 1.5 ml/min. Hydrogen and air flow rates were 30 and 300 ml/min, respectively. The injection volume size was 1 μ l of the prepared samples.

RESULTS AND DISCUSSION

PHB production was carried out in batch fermentation by *H. pseudoflava*. Glucose, fructose and molasses were employed to provide the desire concentrations of substrate in the fermentation media. The optimum bacterial growth leads to optimum PHB accumulation which was dependent on the composition of the fermentation media. Growth kinetic data for *H. pseudoflava* was obtained. The kinetic parameters for the PHB production with various carbon sources are summarized in Table 1.

Figure 1 shows glucose consumption and PHB production and CDW with respect to 96 h incubation time. At optimum conditions 30°C, agitation rate of 250 rpm and incubation period of 48 h, maximum PHB concentration of 2.65 g/l was obtained.

Fructose as a carbon source was experimented for PHB production. At media optimum conditions and incubation time of 96 h, maximum PHB concentration of 1.6 g/l was obtained (Figure 2). Figure 3 shows Malthus kinetic model was well fitted with the experimental data. The fitted line for fructose and glucose were very close to each other. This graph corresponds to Equation 3 which represents the variation of the logarithm of cell concentration with respect to incubation time.

The Lineweaver-Burk plot, double reciprocal plot for Monod kinetic model is shown in Figure 4. The model was used based on experimental data obtained for substrate consumption with respect to incubation time. There was a good agreement between experimental data. The rate data for glucose was more promising while maximum PHB production rate (0.28 g/lh) was obtained. The growth pattern for the microorganism was exactly followed by Logistic model, as the fitted data are presented in Figure 5. Maximum cell concentration was about 18 g/l for fructose incubated at 96 h.

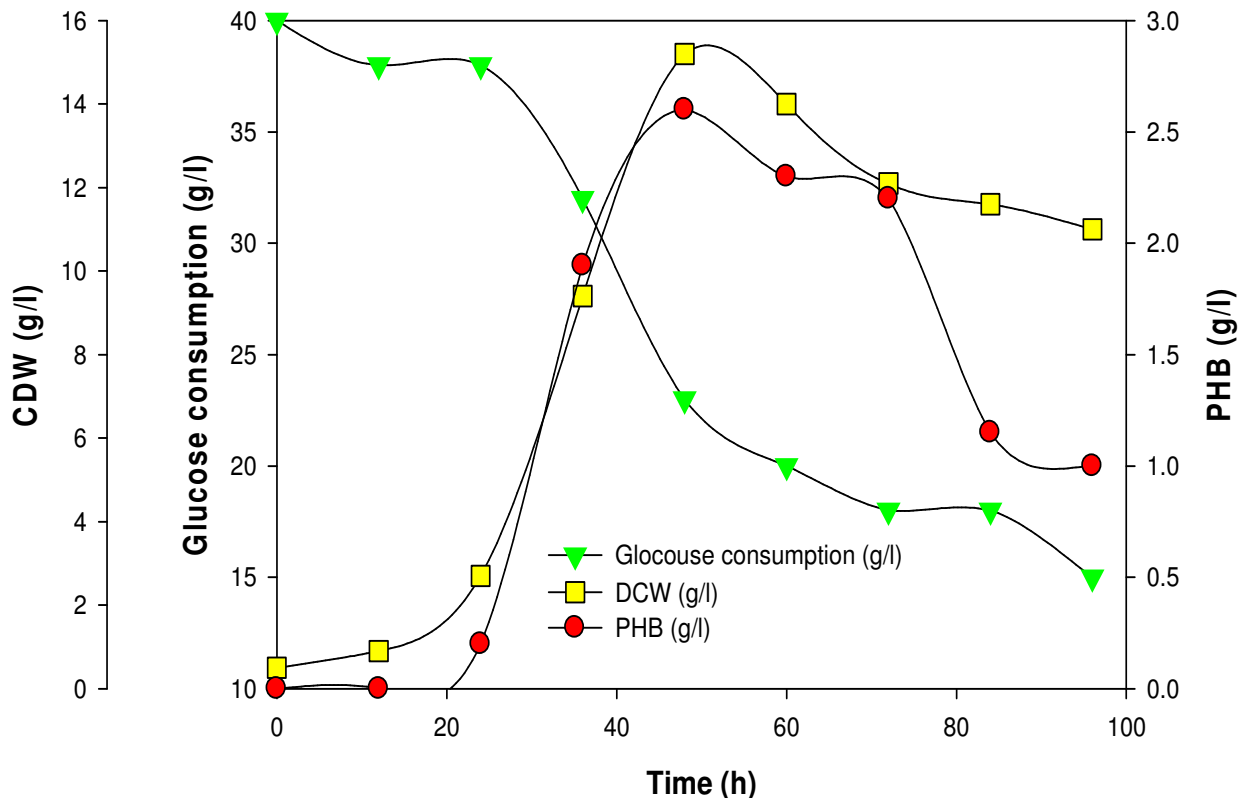


Figure 1. The concentration profiles of cell growth, consumption of glucose and biopolymer (PHB) production at 30°C and agitation rate of 250 rpm.

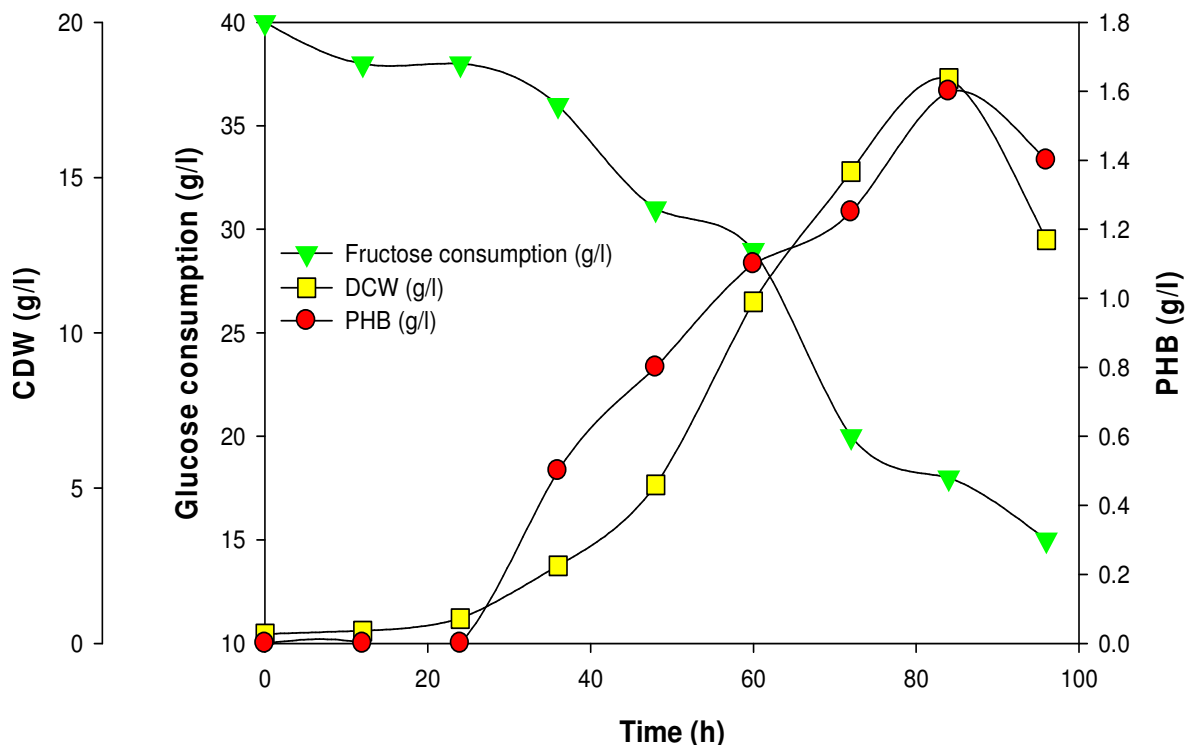


Figure 2. The concentration profiles of cell growth, consumption of fructose and biopolymer (PHB) production at 30°C and agitation rate of 250 rpm.

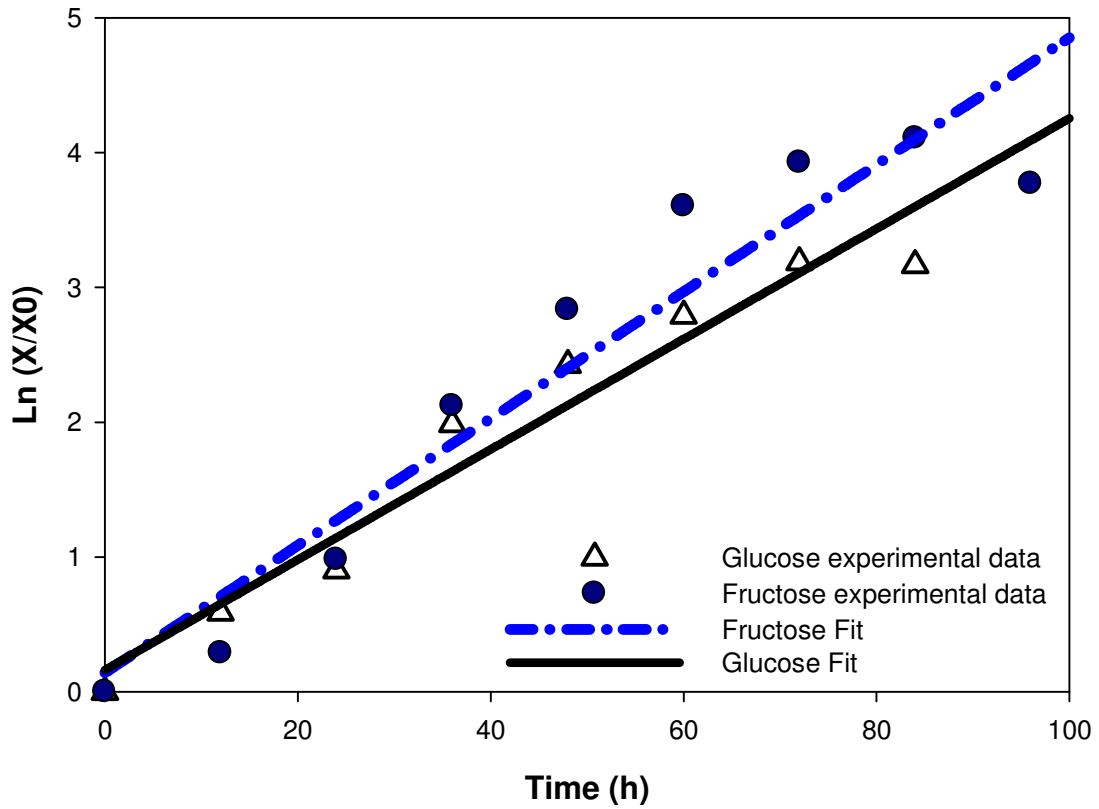


Figure 3. Malthus kinetic model fitted with experimental data.

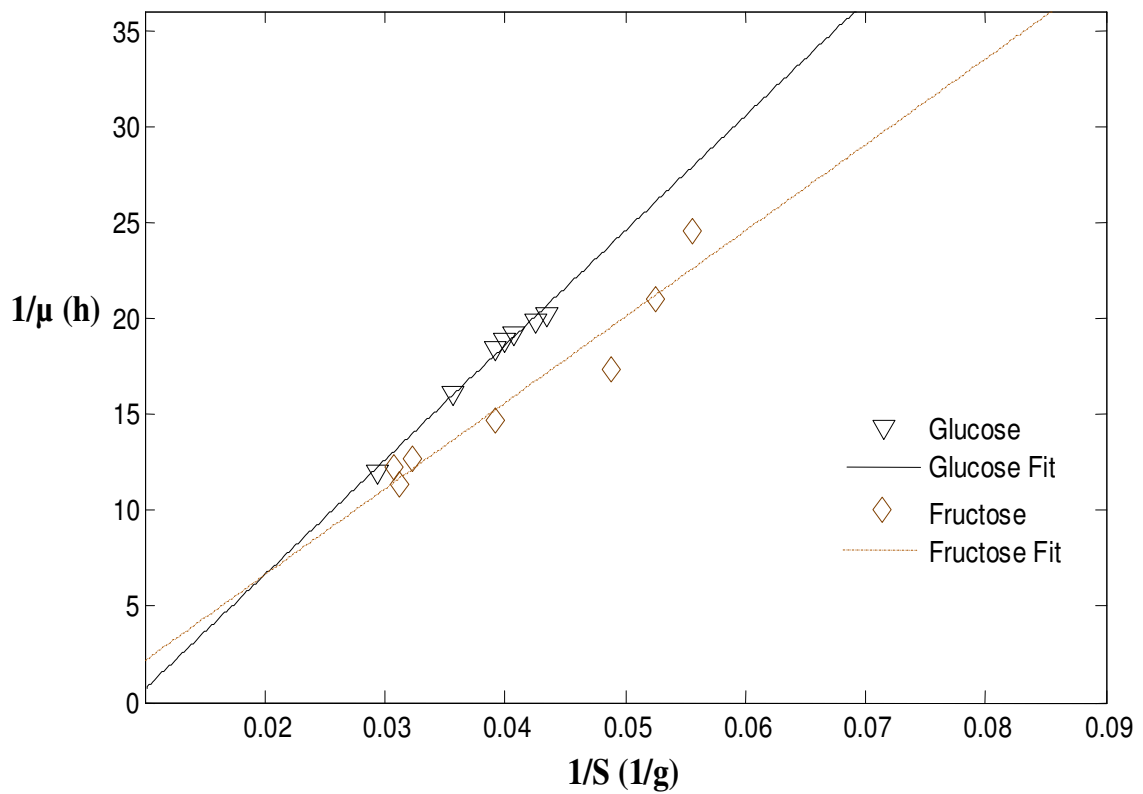


Figure 4. Lineweaver-Burk plot fitted with experimental data.

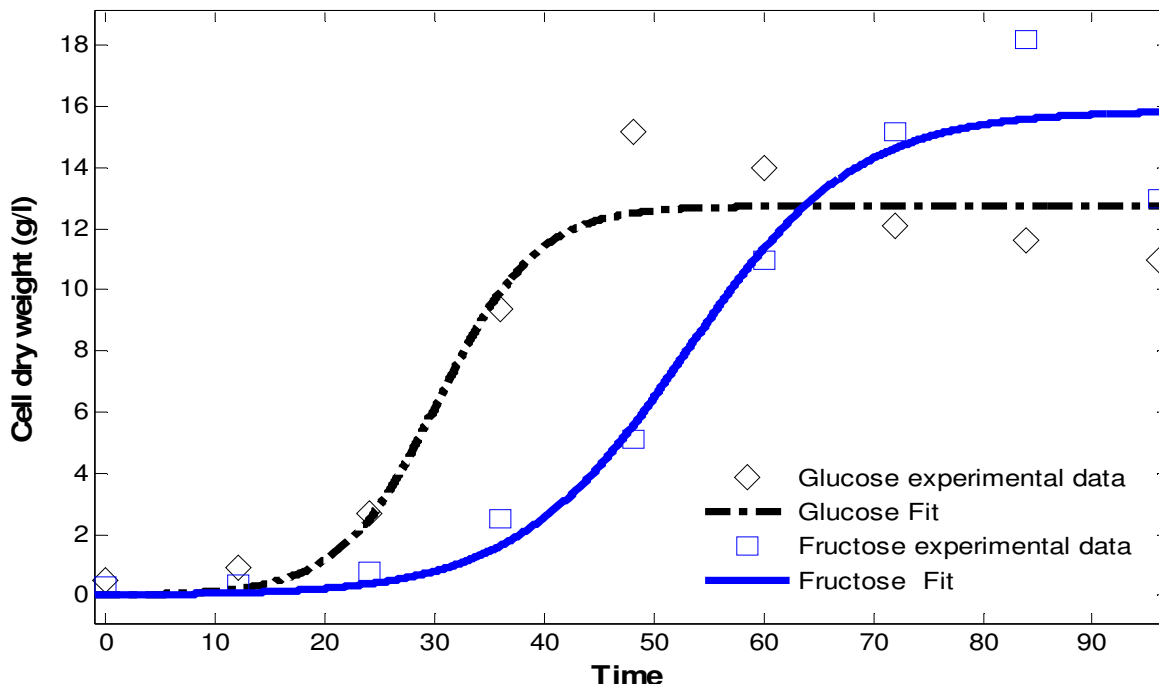


Figure 5. Logistic kinetic model fitted with experimental data.

Conclusion

Mean experimental data of batch cultivation were used to predict kinetic parameters. Among the models and substrate utilized by *H. pseudoflava*, the best model fits well ($R^2 = 0.95$) with glucose utilization by Logistic equation to predict biomass growth. Besides that any inhibition may be predicted by the same model, *H. pseudoflava* showed high growth rate in fructose-based medium as compared to the media contained glucose. In the medium contained glucose as carbon source, the maximum specific growth rate was projected by the Monod kinetic model was obtained. The PHB yield was 0.23 g PHB/g of substrate. It was concluded that the maximum biomass yield was obtained with fructose as carbon source.

ACKNOWLEDGMENT

The authors are grateful to Islamic Azad University – Ayatollah Amoli Branch for financial support of this project.

REFERENCES

- Apostolis AK, Xu Y, Wang R, Webb C (2006). Polyhydroxybutyrate production from a novel feedstock derived from a wheat-based biorefinery. *Enzyme Mic. Technol. J.* 40: 1035-1040.
- Braunegg G, Sonnleitner B, Lafferty RM (1987). A rapid gas chromatographic method for the determination of poly-b-hydroxybutyric acid in microbial biomass. *Eur. J. Appl. Microbiol. Biotechnol.* 6: 22-26.
- Divyashree M, Rastogi N, Shamala T (2009). A simple kinetic model for growth and biosynthesis of polyhydroxyalkanoate in *Bacillus flexus*. *New Biotechnology* 26: 92-98.
- Doi Y, Steinbüchel A (2001). Biopolymer: polyesters II-properties and chemical synthesis. Wiley-VCH.
- Fabiane CO, Marcos LD, Leda RC, Denise MGF (2007). Characterization of poly(3-hydroxybutyrate) produced by *Cupriavidus necator* in solid-state fermentation. *Bioresour. Technol. J.* 98: 633-636
- Ghaly EA, Tango SA, Adams MA (2003). Enhanced Lactic Acid Production from Cheese Whey with Nutrient Supplement Addition. *Agricultural Engineering International the CIGR J. Sci. Resand Dev.* pp. 5-10.
- Gouda MK, Swellam AE, Omar SH (2001). Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen source. *Microbiol. Res. J.* 156: 201-204.
- Khanna S, Srivastava AK (2005). Statistical media optimization studies for growth and PHB production by *Ralstonia eutropha*. *Proc. Biochem.* 40: 2173-2176.
- Kim BS (2000). Production of poly(3-hydroxybutyrate) from inexpensive substrates. *Enzyme Microbiol. Technol. J.* 27: 774-778
- Kumar A, Shamala T, Kshama L, Prakash M, Joshi G, Chandrashekar A, Kumari K, Divyashree M (2007). Bacterial synthesis of poly(hydroxybutyrate-cohydroxyvalerate) using carbohydrate rich mahua (*Madhuca* sp.) flowers. *J. Appl. Microbiol.* 103: 204-209.
- Labuzek S, Radecka I (2001). Biosynthesis of PHB tercopolymer by *Bacillus cereus* UW 85. *J. Appl. Microbiol.* 90: 353-359.
- Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31: 426-429
- Morinaga Y, Yamanaka S, Ishizaki A, Hirose Y (1978). Growth characteristics and cell composition of *Alcaligenes eutrophus* in chemostat culture. *Agric. Biol. Chem.* 42: 439-443.
- Najaf Pour GD (2007). *Biochemical Engineering and Biotechnology*. Elsevier, Amsterdam. Valappil
- Sharifzadeh Baei M, Tabandeh F, Yunesi H, Najafpour G, Issazadeh H (2009). Optimization Phas production by *Cupriavidus necator* from molasses and acetate as substrate. *New Biotechnol.* 25(1): 268-272.

Sharifzadeh Baei M, Tabandeh F, Yunesi H, Najafpour G, Issazadeh H (2009). Poly (3-hydroxybutyrate) synthesis by *Cupriavidus necator* DSMZ 545 utilizing various carbon sources, *World Appl. Sci. J.* 7(2): 157-161.

Sims R (2003). Climate Change solutions from Biomass, Bioenergy and Biomaterials. *Agricultural Engineering International: the CIGR. J. Sci. Res. dev.* Invited Overview. V. September.

Valappil S, Peiris D, Langley G, Herniman J, Boccaccini A, Bucke C, Roy I (2007). Polyhydroxyalkanoate (PHA) biosynthesis from structurally unrelated carbon sources by a newly characterized *Bacillus* spp. *J. Biotechnol.* 127: 475-480.