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Kinetics Evaluation of Cell Growth and PHB Production by *Azotobacter beijerinckii* DSMZ 1041

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Abstract: In this research a kinetic model for cell growth evaluation and biopolymer production by *Azotobacter Beijerinckii* DSMZ 1041 on different carbon sources were conducted. Logistic model, Malthus model and Luedeking-Piret model equation were used for kinetic parameters prediction. A good agreement was found between the experimental and predicted values in glucose consumption as carbon source in compare of two other carbon sources.The specific growth rates μ_m of Logistic model for glucose, fructose and whey were 0.155,0.197 and 0.112 respectively. The specific growth rates μ_m of Malthus model for glucose, fructose and whey were 0.02,0.035 and 0.011 respectively. and growth coefficient (α,β) for glucose,fructose and whey were deliberated.

Key words: Kinetic model · PHB · Logistic model · Malthus model · Luedeking-piret model

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are naturally biodegradable polyesters produced by a wide variety of microorganisms [1]. They can Be composed of over 100 different monomers. Thus, the variation in PHA composition provides a wide range of mechanical integrity and biochemical properties. In the last decade, PHAs have attracted A great deal of attention for their potential use in the development of Tissue engineering biomaterials, as they have particular properties.

That are not available in existing synthetic polymers Among PHAs, the most intensively investigated is poly (3-hydroxybutyrate), a natural compound of blood and tissue [2]. However, unrefined carbon sources such as corn syrup, sugarcane molasses, beet molasses, or malt extract are utilized for PHB formation. It was reported that, PHB yield with various untreated carbon sources were even better than the refined carbohydrate sources [2]. Beet molasses and malt extract promoted high polymer production due to presence of a growth stimulants in the cultured media [2]. PHB production by number of wild type bacterial strains occurs under nutrient depletion conditions [3]. In PHB production phase, the cell growth is limited due to depletion of essential nutrients such as carbon, nitrogen and phosphorus sources. Such depletion in the presence of excess amount of carbon source triggers the metabolic shift from growth to PHB production modes. It was also reported that *C. necator*,which is known as *Wautersia eutropha*, *Ralstonia eutropha* and *Alcaligenes eutrophus* are potential organisms for the optimal production of PHB, that is a homopolymer which is accumulated inside the cells under nitrogen limitation [3-4]. *Azotobacter beijerinckii* produces appreciable amount of PHB, the biopolymer concentration increases under oxygen limitation. The organism may tolerate nitrogen and phosphorus limitation [5]. A wide variety of PHA copolymers are synthesized by number of microorganisms via fermentation processes while utilizing various carbon sources [6-8]. PHB production is a complex process; where the final quality and quantity of the product yield depends on strains, metabolic pathway, process fermentation parameters, PHB production phase (growth and stationary phases), carbon sources and nutrient depletion conditions which are required for PHB synthesis.

Useful kinetic model for biopolymer synthesis has been implemented including biomass (cell density), product concentration, single substrate utilization and

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limited nutrient sources [9-10]. In a biopolymer process, the kinetic model is substantially capable to predict product formation. Mathematical models facilitate data analysis and provide a strategy for solving problems encountered in fermentation processes. Information on fermentation process kinetics is potentially valuable for the improvement of process performances. Kinetic model has the potential to approximate and allows us to predict the cell growth may contain biopolymer. One of the most widely used model to describe cell growth known as unstructured model that describes the single component as the sole source of energy for prediction of cell growth. It is common to assume ideal case that is single substrate and the rate is defined by Monod growth rate model [11-12]. Similarly, rate models are successfully used to estimate kinetic parameters for growth expression in PHB production by *W. eutropha*, which is subsequently used in modeling the cell growth and biopolymer production [13-14]. In another investigation by Dhanasekar *et al.* [15] Monod, logistic and modified logistic models were successfully applied to describe the batch growth kinetics.

At the end of the lag phase, the growth of microorganisms well adjusted to its new environment. Then the cells multiply rapidly. 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 [10].

The Leudiking-piret model used for PHBs production described by the following equation:

$$\frac{dp}{dt} = a\frac{dx}{dt} + \beta x \tag{1}$$

Where P is the product cocenteration (g/l), X is cell concentration (g/l) and the terms α and β are defined as related and unrelated growth coefficients, respectively.

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 [10]:

$$\mu = \frac{1}{x}\frac{dx}{dt} \tag{2}$$

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

$$Ln\frac{x}{x_0} = \mu t \tag{3}$$

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 [12]. The cell growth rate was evaluated based on growth kinetics. Logistic equation was a suitable kinetic model for prediction of growth curve. The Logistic equation is a substrate independent rate model, which is used for the determination of inhibition effect on biomass growth. It was theoretically proposed that the inhibition factor was proportional to microbial biomass growth [3]. The specific growth rate is predicted by Logistic model presented by equation 4.

$$\mu = \mu_m(1 - \frac{X}{X_m}) \tag{4}$$

Where X_m 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 [16]:

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

The above equation was used to predict the cell growth in batch experiments. In this research, inoculation volumes were kept constant for batch experiments. Matlab (V 7.1) computer software was used to define logistic growth kinetic parameters.

The main purpose of present research was to investigate the effect of various carbon sources such as glucose, fructose and whey on biopolymer production. In addition to find a suitable cheap carbon source for PHB production. Kinetic parameters for the cell growth and PHB production by *Azotobacter beijerinckii* were determined.

MATERIALS AND METHODS

Microorganism: *Azotobacter beijerinckii* DSMZ 1041 was used in all experiments. The strain was maintained on agar slants and petridishes stored at 4°C. Also for revival of the organism, the culture was monthly renewed.

Media

Batch Culture Study: A mineral salt medium which consisted of: 1.0 g/l $(NH_4)Cl$, 2.3 g/l KH_2PO_4, 2.9 g/l K_2HPO_4, 1 g/l $MgSO_4.7H_2O$, 10 mg/l $CaCl_2$, 50 mg/l, Fe (NH_4), 0.5 g/l $NaHCO_3$, 5 ml/l trace metal solution, 0.5 g/l yeast extract and 1.0 g/l peptone. The trace metal solution consisted of: 2.2 g/l $MgSO_4.7H_2O$, 0.1 g/l $FeSO_4.5H_2O$, 0.08 g/l Zn $SO_4.7H_2O$, 2.2 g/l K_2SO_4, 0.02 g/l H_3BO_3, 0.08 g/l $CuSO_4$. Glucose was used as carbon source with concentration of 40 g/l for preparation of seed culture and media used as inoculums. Glucose, yeast extracts and peptone, K_2HPO_4, Fe (NH_4), $NaHCO_3$ and trace metal solution were sterilized separately at 121°C and then aseptically reconstituted at room temperature prior to inoculation.

The media used for comparison studies with variation of carbon and nitrogen sources concentration has the same composition except the described variable term varied. With concentration variation of $(NH_4)Cl$ and glucose as nitrogen and carbon sources media were prepared for these sets of experiments.

Experimental Method: Mineral salt medium containing 40g/l glucose used for inoculums development. The organism was cultivated in a 250 ml Erlenmeyer flask containing 50 ml of the medium incubated in an incubator shaker (IKA, Germany) at agitation rate of 250 rpm and 30°C for 15 h. Medium initial pH was set to 7.0. For production of PHB, 100 ml of medium was taken in a 500 ml flask and inoculated with 5 ml of inoculum. The flask was incubated under agitation conditions for 72 h at 250 rpm and 30°C. Samples were withdrawn at regular defined time intervals and analyzed for biomass and PHB accumulation and residual glucose.

Analytical Methods: Cell Dry weight: The cell concentration of the cultured media was determined by the cell optical density at a wavelength of 620 nm with the aid of a spectrophotometer (UNICO2100, USA) after suitable dilution with distilled water. The cell dry weight was also measured based on standard calibration curve of absorbance as a function of cell dry weight for the pure culture of *A. beijerinckii* [3, 17].

Biopolymer extraction: Extraction of biopolymer was conducted according to the method developed by Braunegg [18-19]. For biopolymer quantification, a 5 ml of culture broth was centrifuged at 3600 rpm for 20 min. A solution of 2 ml of chloroform and 2 ml of acidified methanol (3% sulfuric acid) were added to the cell pellet in vial with Teflon screw cap and heated at 100°C for 3.5 h.

Determination of total carbohydrate concentration: Supernatant was used for residual nutrient analysis including total sugar. The method is based on total reducing sugar by reagent of dinitrosalicilic acid (DNS) method [20]. Standard method was developed using the reagent for colorimetric method to detect orange color using spectrophotometer at wavelength of 580 nm.

Biopolymer analysis: Gas chromatography (GC) was performed by 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), used for the methyl-3- hyroxybutyrate (3HB) analysis. The GC column, capillary column (BP20 SGE, Australia) with 0.33 mm internal diameter, 25 m length was used. The column temperature was initially maintained at 80°C for 4 min, followed by a temperature program 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 gases used were helium as a carrier gas with a flow rate of 1.5 ml/min, hydrogen 30 ml/min and air 300 ml/min. The injection volume size was 1µl of the prepared samples [3, 21].

RESULTS AND DISCUSSION

In current research, logistic and malthus models were used for prediction of cell growth and Leudeking-Piret employed for production. Also growth kinetic parameters evaluated from kintics models.

A combination of logistic and malthus equations are employed for effective microorganism growth estimation. For this Issue, logistic model used for initial growth to stationary phase (stage10 and malthus model employed for death phase to end of process(stage2). comparison of experimental and model results are shown in Figure 1, figure 2, figure 3. Combinated model for two stages of growth led to a good agreement between experimental data and model results.

For production kinetic evaluation for stage 1, logistic equation (5) substitute in Leudiking-piret model (1) as follow:

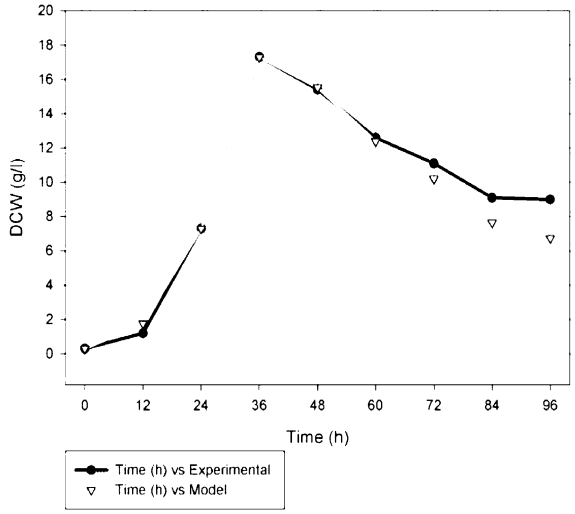

Fig. 1: Dry cell weight for glucose as sole carbon source

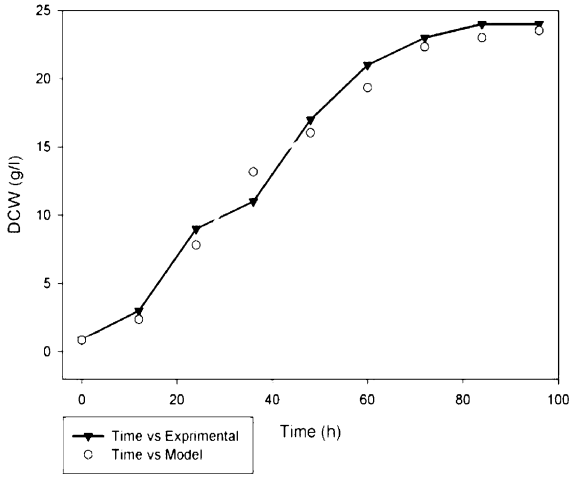

Fig. 2: Dry cell weight for fructose as sole carbon source

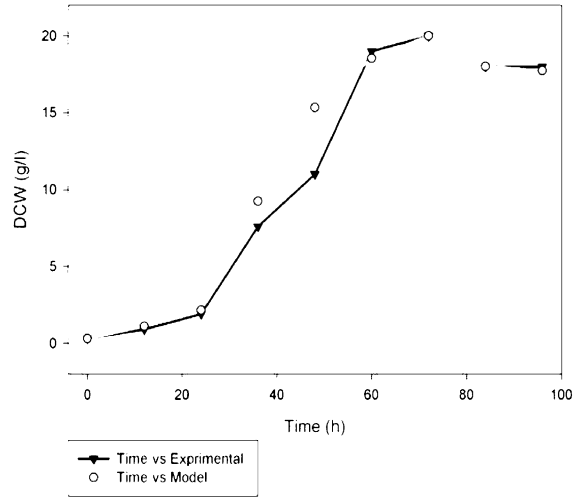

Fig. 3: Dry cell weight for whey as sole carbon source

$$\left\{P(t) = P_0 + \alpha x_0 \left\{\frac{exp(\mu_m t)}{\left[1-\left(\frac{x_0}{x_m}\right)(1-\exp(\mu_m t))\right]} - 1\right\}\right. \quad (6)$$

$$\left. + \beta \frac{x_m}{\mu_m} ln\left[1-(\frac{x_0}{x_m})(1-\exp(\mu_m t))\right]\right.$$

Also the above equation (6) can be change to:

$$P(t) = P_0 + \alpha A + \beta B \quad (7)$$

In stationary phase $\frac{dx}{dt} = 0$ and $x=x_m$, so equation (1) is changed to:

$$\beta = \frac{\frac{dp}{dt}(stationary\ phase)}{x_m} \quad (8)$$

Also α can be evaluated as slop of verses A (t).

For stage 2, the Leudeking-piret equation (1) has been changed by substituting Malthus equation as follow:

$$P(t) = P_0 + \alpha x_0 \exp(\mu.t) + \beta \frac{x_0}{\mu} \exp(\mu.t) = \alpha A(t) + \beta B \text{ verses A (t).} \quad (9)$$

Therefore, we can stimulate biopolymer production under Batch condition by *A. Beijerinckii*.

For glucose, fructose and whey as sole carbon sources. Figures 4, 5 and 6 illustrate biopolymer comparison of experimental data and model predictions for biopolymer production from glucose, fructose and whey, respectively.

As it mentioned before, logistic model and Malthus model employed for dry cell estimation in in first stage and second stage, respectively. With combination of these two equations, the dry cell weight evaluated during the batch process which was 17.3 g/l.This predicted amount by model was very close to experimental that.so it is clear that the model had a good agreement with experimental data. In same way for biopolymer production, modified Leudeking-piret by logestic and Malthus equations led to good concurrence in compare of experimental data.

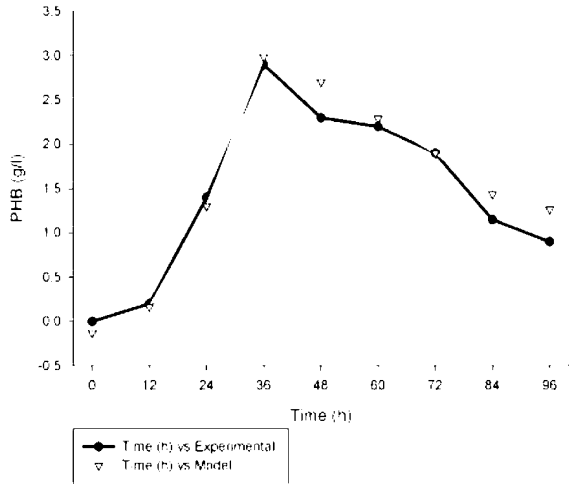

Fig. 4: Biopolymer production by *Azotobacter Beijerinckii* from glucose

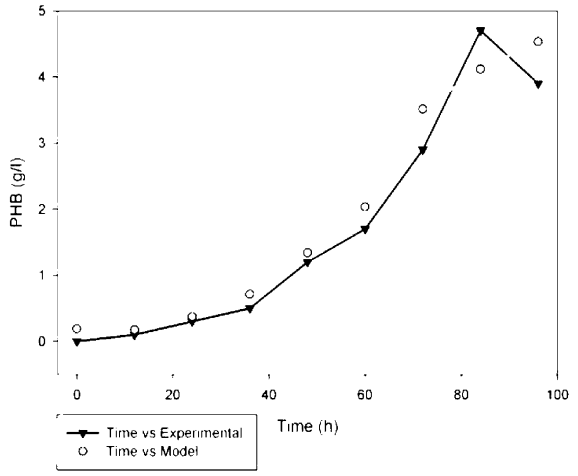

Fig. 5: Biopolymer production by *Azotobacter Beijerinckii* from fructose

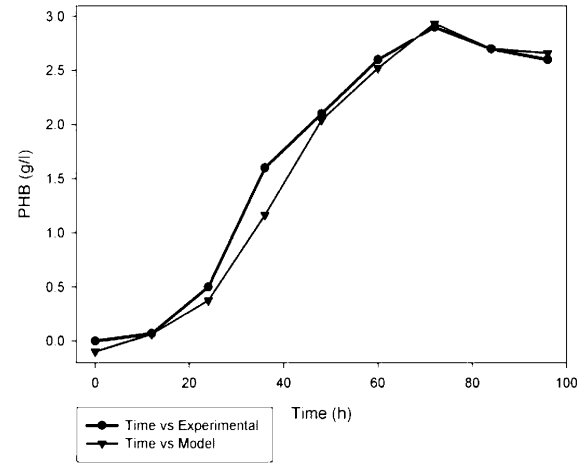

Fig. 6: Biopolymer production by *Azotobacter Beijerinckii* from whey

Table 1: Kinetic parameters for biopolymer production from glucose

Paremeters	Logistic model	Malthus model
μ_m	0.1551	0.0205
α	0.1846	1.076
ß	0.0023	

Table 2: Kinetic parameters for biopolymer production from fructose

Kinetic parameters	Logistic model	Malthus model
μ_m	0.11974	0.0349
α	0.2176	0.3571
ß	0.005	

Table 3: Kinetic parameters for biopolymer production from whey

Kinetic parameters	Logistic model	Malthus model
μ_m	0.112	0.0111
α	0.1708	2.737
ß	0.0020	

According to these suitable stimulation, kinetic parameters for biopolymer production for glucose, fructose and whey are shown in tables 1, 2 and 3 respectively.

CONCLUSION

Growth Kinetic evaluation for biopolymer production has been investigated by several kinetics models. Novelty of present research is combination of kinetic models for better agreement between experimental data and model predictions which were employed in cell growth and biopolymer production prediction. The results showed that this combination led to a good concurrence in experimental and model predicted data.

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