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Fed-batch cultivation of *Wautersia eutropha*

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Abstract

Batch kinetics of polyhydroxybutyrate (PHB) synthesis in a bioreactor under controlled conditions of pH and dissolved oxygen gave a biomass of 14 g l^{-1} with a PHB concentration of 6.1 g l^{-1} in 60 h. The data of the batch kinetics was used to develop a mathematical model, which was then extrapolated to fed-batch by incorporating the dilution due to substrate feeding. Offline computer simulation of the fed-batch model was done to develop the nutrient feeding strategies in the fed-batch cultivation. Fed-batch strategies with constant feeding of only nitrogen and constant feeding of both nitrogen and fructose were tried. Constant feeding strategy for nitrogen and fructose gave a better PHB production rate of 0.56 g h^{-1} over the value obtained in batch cultivation (PHB production rate – 0.4 g h^{-1}).

Keywords: PHB; Modeling; Fed-batch cultivation; *Wautersia eutropha*; Biopolymer

1. Introduction

Plastics are used in almost all industries ranging from electronics to heavy automobiles, especially the packaging industry where it accounts for more than 50% of total consumption. The reason for such a wide usage is their versatile qualities of strength, lightness, durability and resistance to corrosion. Particularly due to the property of resistance to degradation including biodegradation, plastics have become indispensable as packaging materials. However, this property of plastics makes them an environmental hazard (Brandl et al., 1990). In such a scenario, biodegradable plastics that completely mineralize to carbon dioxide, methane and biomass and have properties similar to conventional plastics, offer an attractive alternative to conventional plastics.

Polyhydroxyalkanoates are 100% biodegradable plastics. They are polyesters of various hydroxyalkanoates which are synthesized by numerous microorganisms as energy reserve materials when an essential nutrient such as nitrogen or phosphorus is limited in presence of excess carbon source (Khanna and Srivastava, 2005a). They possess properties similar to various synthetic thermoplastics like polypropylene and hence can be used in their place. They are also completely degraded to water and carbon dioxide under aerobic conditions and to methane under anaerobic conditions by microorganisms in soil, sea, lake water and sewage (Lee, 1996).

Poly (β-hydroxybutyrate) (PHB) is the most widely studied member belonging to this group. PHB production by microorganisms (James et al., 1999; Borah et al., 2002 Dhanasekar et al., 2003; Khanna and Srivastava, 2005b) has been attempted but still there is a need to address the problem of improvement of yield and productivity of PHB so that it can economically compete with the production cost of conventional plastics.

It has been observed (Mulchandani et al., 1988) that nitrogen/carbon ratio (N/C ratio) plays an important role in the fermentation of *W. eutropha* since it affects the overall kinetics of growth and PHB accumulation. PHB accumulation occurs when nitrogen concentration is low and excess of carbon source is available and this condition can be represented in terms of optimum level of N/C. Maintenance of N/C ratio at an optimum level leads to better PHB accumulation but it is difficult to maintain this level by trial and error experimental design of fed-batch fermentation. In a

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Nomenclature

X	total biomass concentration
R	residual biomass concentration
P	product concentration
S_N	nitrogen concentration
S_F	fructose concentration
RR	rate of formation of R
RP	rate of formation of P
RSN	rate of consumption of nitrogen
RSF	rate of consumption of fructose
K_{S1}	saturation constant for monod growth term in Eq. (3)
K_{S2}	saturation constant for sigmoidal growth term in Eq. (3)
n	exponential constant for sigmoidal growth term in equation
m	exponent in inhibition term in Eq. (3)
S_m	ratio of N/C at which specific growth rate is zero
K_1	constant for non-growth associated term in Eq. (4)
R_{min}	minimum value of R at which PHB accumulation starts
K_2	constant for inhibition due to high product concentration in Eq. (4)
K_S	constant in Eq. (4)
e	kinetic parameter for growth associated term in Eq. (4)
a, b, c	kinetic parameters in Eq. (5)
f	kinetic parameter in Eq. (6)
V	volume
F_1	nitrogen flow rate
F_2	fructose flow rate
F	total flow rate
S_{N0}	nitrogen concentration in feed
S_{F0}	fructose concentration in feed
Greek letters	
μ_{m1}	maximum specific growth rate for monod growth term in Eq. (3)
μ_{m2}	maximum specific growth rate for sigmoidal growth term in Eq. (3)
μ_R	rate at which residual biomass is formed

model based approach, offline computer simulations can be done to predict the (nitrogen/fructose) feeding strategies for desired biomass formation and PHB accumulation prior to actual experimentation and only those feeding strategies which show an improvement over the batch in terms of biomass/PHB accumulation can be implemented. This approach has been attempted in the present study.

2. Methods

2.1. Maintenance of culture

The bacterial strain used in the present study was *W. eutropha* NRRL B-14690 (earlier known as *Ralstonia eutropha* and *Alcaligenes eutrophus*). It was maintained on Luria Agar slants at 4 °C and was sub-cultured every 15 days to maintain its viability.

2.2. Media

The media for *W. eutropha* cultivation as reported in the literature was used in this investigation (Raje and Srivastava, 1998). In all cases, fructose and rest of the media components were sterilized separately and mixed aseptically before inoculation.

2.3. Batch kinetics studies

The culture conditions were same as reported in our earlier studies (Patwardhan and Srivastava, 2004). Four litre of media was taken in 7 l Bioengineering (Bioengineering AG, Switzerland) bioreactor, sterilized in situ and inoculated with 5% inoculum. Dissolved oxygen concentration was measured using an in situ Ingold (Ingold, Switzerland) dissolved oxygen probe and maintained at 30% saturation value by manually adjusting the speed of the agitator and flow of sterile air. Samples were taken at regular intervals for estimation of biomass, residual nutrients and PHB.

2.4. Fed-batch fermenter studies

The fermenter was run as batch till 17 h after which the feeding of nitrogen/fructose was started as per the various nutrient feeding strategies predicted by the model.

2.5. Analytical methods

Optical density (OD) of the suitably diluted cell suspension was measured at 600 nm against a medium blank in a spectrophotometer (Spekol 1200, Analytik Jena, Germany). The sample was centrifuged (Sorvall RC5B Centrifuge) at 10,000 rpm for 10 min and the supernatant was taken for analysis of residual fructose, nitrogen and phosphate. The analytical methods were similar to those reported in our earlier studies (Patwardhan and Srivastava, 2004).

3. Results and discussion

3.1. Cultivation of W. eutropha in bioreactor

Three batch experiments were carried out and the average values of biomass, PHB and residual nutrients are plot-

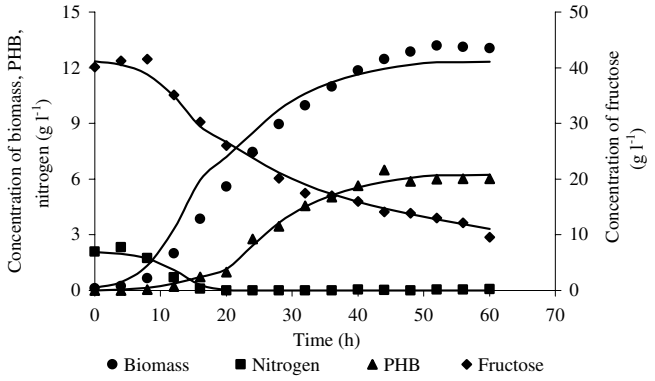

Fig. 1. Batch fermentation kinetics of *W. eutropha*. Comparison of experimental and model simulated values. (Solid lines represent the model simulated values; dots represent the experimental values.)

ted in Fig. 1. The culture entered the exponential phase after a lag of 4–5 h. Ammonium sulphate was completely consumed in 20 h. Intracellular PHB accumulation was induced from 20 h. No phosphate limitation was observed in the batch cultivation (data not shown).

Maximum biomass and PHB concentration of 14 g l^{-1} and 6.1 g l^{-1} was obtained at the end of the fermentation, respectively, with a consumption of 31.5 g l^{-1} fructose. Biomass yield to fructose ($Y_{X/S}$) was 0.44, the product yield to fructose ($Y_{P/S}$) was 0.19 and maximum specific growth rate (μ) was 0.28 h^{-1}. PHB content of the cells was 43.6%.

3.2. Modeling

The model proposed by Raje and Srivastava (1998) was used in the present study.

Basic assumptions of the model were:

1. Biomass (X) is structured as having two components
   a. The catalytically active component consisting of proteins, nucleic acids and cell wall (R).
   b. The inert component which is the product PHB (P).
2. Nitrogen is the limiting substrate affecting the growth kinetics.

3.2.1. Batch model

Based on the assumptions, total biomass is defined as

$$X = R + P \tag{1}$$

where R (residual biomass) is the catalytically active growth component.

Rate of formation of residual biomass (R) is given as

$$\mathrm{R}R = \frac{\mathrm{d}R}{\mathrm{d}t} = \mu_{\mathrm{R}} R \tag{2}$$

The specific growth rate equation featured nutrient limitation by both monod and sigmoidal growth terms. The inhibition due to N/C ratio (studied by Mulchandani et al., 1988) was also incorporated in the model structure:

$$\mu_{\mathrm{R}} = \left[\mu_{\mathrm{m1}}\frac{S_{\mathrm{N}}}{K_{\mathrm{S1}}+S_{\mathrm{N}}} + \mu_{\mathrm{m2}}\frac{\left(\frac{S_{\mathrm{N}}}{K_{\mathrm{S2}}}\right)^{n}}{\left[1+\left(\frac{S_{\mathrm{N}}}{K_{\mathrm{S2}}}\right)^{n}\right]}\right]\cdot\left[1-\left(\frac{S_{\mathrm{N}}/S_{\mathrm{F}}}{S_{\mathrm{m}}}\right)^{m}\right] \tag{3}$$

The rate of formation of product was assumed to have growth and non-growth associated components, and inhibition due to high product concentration:

$$\mathrm{R}P = \frac{\mathrm{d}P}{\mathrm{d}t} = [K_1(R-R_{\min}) - K_2 P]\frac{K_{\mathrm{S}}}{K_{\mathrm{S}}+S_{\mathrm{N}}} + e\frac{\mathrm{d}R}{\mathrm{d}t} \tag{4}$$

Fructose consumption rate was assumed to be due to growth of R, formation of P and maintenance (proportional to R):

$$\mathrm{RSF} = \frac{\mathrm{d}S_{\mathrm{F}}}{\mathrm{d}t} = -\left[a\frac{\mathrm{d}R}{\mathrm{d}t} + b\frac{\mathrm{d}P}{\mathrm{d}t} + cR\right] \tag{5}$$

Nitrogen was consumed due to the growth of R:

$$\mathrm{RSN} = \frac{\mathrm{d}S_{\mathrm{N}}}{\mathrm{d}t} = -f\frac{\mathrm{d}R}{\mathrm{d}t} \tag{6}$$

The model parameters were identified by minimizing the difference between the experimental data and the model simulations by the computer programs and methodology described in our earlier works (Patwardhan and Srivastava, 2004).

3.2.2. Fed-batch model

The batch model was extrapolated to fed-batch cultivation by incorporating the dilution terms. The fed-batch model equations are given below:

$$\frac{\mathrm{d}V}{\mathrm{d}t} = F_1 + F_2 = F \tag{7}$$

$$\frac{\mathrm{d}R}{\mathrm{d}t} = \mathrm{R}R - \frac{F}{V}R \tag{8}$$

$$\frac{\mathrm{d}P}{\mathrm{d}t} = \mathrm{R}P - \frac{F}{V}P \tag{9}$$

$$\frac{\mathrm{d}S_{\mathrm{N}}}{\mathrm{d}t} = RS_{\mathrm{N}} + \frac{F_1 S_{\mathrm{N0}}}{V} - \frac{FS_{\mathrm{N}}}{V} \tag{10}$$

$$\frac{\mathrm{d}S_{\mathrm{F}}}{\mathrm{d}t} = RS_{\mathrm{F}} + \frac{F_2 S_{\mathrm{F0}}}{V} - \frac{FS_{\mathrm{F}}}{V} \tag{11}$$

where F_1 and F_2 are the flow rates for nitrogen and fructose feed, respectively. Various fed-batch strategies were simulated for growth enhancement and improved intracellular product concentration. Two such strategies were selected and experimentally verified.

3.3. Fed-batch studies

3.3.1. Constant feeding of nitrogen without fructose feeding (Fig. 2)

In batch cultivation, it was observed that nitrogen was totally consumed in 20 h, however, 28 g l^{-1} of fructose

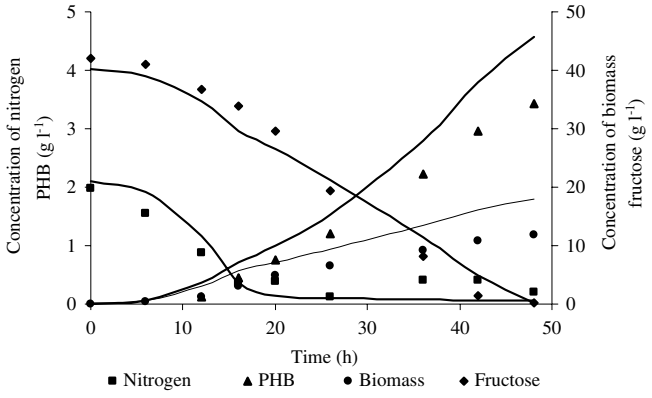

Fig. 2. Biomass, PHB and residual nutrient profiles for fed-batch strategy (constant rate of nitrogen feeding (ammonium sulphate, 60 g l^{-1}) at 0.007 l h^{-1} without fructose supplementation). (Solid lines represent the model simulated values; dots represent the experimental values.)

was still available. A fed-batch was therefore, designed (using the mathematical model) to supplement the nitrogen concentration and enhance the biomass accumulation. Several feeding strategies were simulated in which the concentration of nitrogen was varied from 10 g l^{-1} to 100 g l^{-1}. Also the rate at which the feeding solution was added varied from 0.002 l h^{-1} to 0.01 l h^{-1}. However, among all the strategies simulated best results were obtained when nitrogen feeding was done at a flow rate of 0.007 l h^{-1} with a feeding solution containing 60 g l^{-1} ammonium sulphate.

A simple constant feeding of nitrogen was attempted, starting from the time when nitrogen concentration approached zero level in batch fermentation. 60 g l^{-1} of ammonium sulphate was fed at a constant rate of 0.007 l h^{-1} from 17 h until the end of the fermentation (when fructose concentration reached zero). The feeding of nitrogen was done to increase the biomass so that PHB accumulation was induced in higher amount of biomass. The computer simulation also predicted that fructose (initial concentration of 40 g l^{-1}) was completely consumed in 48 h. As can be seen from Table 1, the model prediction featured higher biomass production rate of 1.39 g h^{-1} as opposed to 0.9 g h^{-1} in batch cultivation and PHB production rate of 0.39 g h^{-1}, which was comparable to 0.4 g h^{-1} for batch cultivation.

This strategy predicted higher biomass production with a very simple feeding profile i.e., with just the feeding of nitrogen and was experimentally implemented. It served to check the validity of the predicted model as well. Maximum biomass and PHB obtained at the end of 48 h were 12.9 g l^{-1} and 3.4 g l^{-1} as compared to predicted values of 15.9 g l^{-1} and 4.5 g l^{-1} as shown in Fig. 2. Total biomass production rate was 1.12 g h^{-1} and total PHB production rate was 0.29 g h^{-1} as shown in Table 1.

3.3.2. Constant feeding of nitrogen with fructose feeding (Fig. 3)

During the simulation of previous fed-batch cultivation, it was observed that with constant feeding of 60 g l^{-1} nitrogen at 0.007 l h^{-1} starting from 17 h, till end of fermentation, fructose was completely consumed in 48 h. It was therefore assumed that if fructose was supplemented at 48 h or earlier, then with the availability of nitrogen and fructose, PHB accumulation may continue for longer period of time. Therefore, fructose feeding of 350 g l^{-1} at 0.015 l h^{-1} was also initiated at 36 h when fructose concentration dropped to 8 g l^{-1} and previously initiated feeding of 60 g l^{-1} nitrogen at 0.007 l h^{-1} was continued. This feeding strategy ensured low nitrogen availability and low non-limiting fructose concentration in the broth, which was favorable for PHB production as can be seen from the high values of total biomass production rate (1.95 g h^{-1}) and total PHB production rate (0.73 g h^{-1}) predicted by the model simulation. Maximum biomass and PHB concentration of 22.6 g l^{-1} and 8.2 g l^{-1} were obtained in 72 h compared to predicted values of 28.6 g l^{-1} and 10.6 g l^{-1} respectively as shown in Fig. 3. Total biomass production rate was 1.54 g h^{-1} and PHB production rate was 0.56 g h^{-1}.

Batch fermentation using fructose as a carbon source and ammonium sulphate as nitrogen source has been studied by Mulchandani et al. (1988). Biomass concentration of 6.6 g l^{-1} and PHB accumulation of 3.8 g l^{-1} was obtained with productivity of 0.078 g l^{-1} h^{-1}. In the present study, batch fermentation of *W. eutropha* NRRL B-14690 resulted in biomass and PHB concentration of 14.0 g l^{-1} and 6.1 g l^{-1} leading to a productivity of 0.09 g l^{-1} h^{-1}.

Various researchers have studied fed-batch fermentation for PHB production. Fed-batch fermentation of glucose utilizing mutant of *W. eutropha* using nitrogen limitation (Kim et al., 1994) yielded biomass and PHB concentration

Table 1
Comparison of batch and fed-batch cultivations

Cultivation strategy	Biomass (g l^{-1})		PHB (g l^{-1})		Final volume (l)	Biomass production rate (g h^{-1})		PHB production rate (g h^{-1})	
	Pred*	Exp**	Pred*	Exp**		Pred*	Exp**	Pred*	Exp**
Batch	13.0	14.0	6.2	6.1	4.0	0.86	0.9	0.41	0.4
Fed-batch strategy without fructose supplementation	15.9	12.9	4.5	3.4	4.2	1.39	1.12	0.39	0.29
Fed-batch strategy with fructose supplementation	28.6	22.6	10.6	8.2	4.93	1.95	1.54	0.73	0.56

* Model predicted values.
** Experimental values.

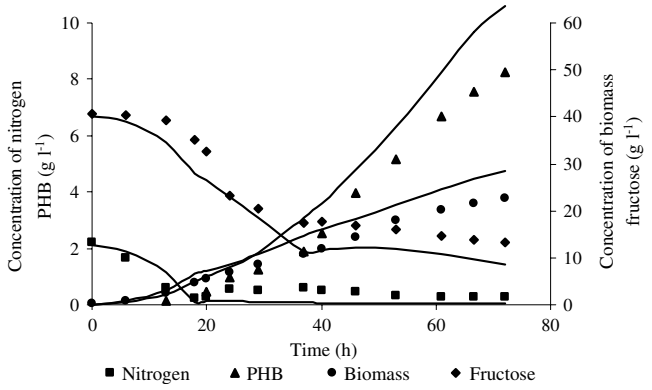

Fig. 3. Biomass, PHB and residual nutrient profiles for fed-batch strategy (constant rate of nitrogen feeding (ammonium sulphate, 60 g l^{-1}) at 0.007 l h^{-1} starting at 17 h with fructose supplementation (350 g l^{-1}) at 0.015 l h^{-1} starting at 36 h). (Solid lines represent the model simulated values; dots represent the experimental values.)

of 164 g l^{-1} and 121 g l^{-1} respectively after 50 h leading to a productivity of 2.42 g l^{-1} h^{-1}. Here, glucose concentration was maintained between 0 and 20 g l^{-1} using on line enzymatic glucose analyzer. But particularly in a large-scale cultivation it is difficult to use such enzymatic sensors for control of substrate concentration.

A two-stage cultivation method has also been used to produce PHB (Tanaka et al., 1995) using *W. eutropha* ATCC 17697 (equivalent to NRRL B-14690 used in the present study). In the first stage culture was grown on 10 g l^{-1} fructose as carbon source followed by autotrophic culture (where a mixture of CO_2, H_2 and O_2 was used for PHB accumulation). PHB concentration of 21.6 g l^{-1} was obtained. Use of acetic acid instead of fructose in the two-stage method has also been investigated (Sugimoto et al., 1999). Acetic acid concentration was maintained at 1 g l^{-1} by pH stat feeding of acetic acid. Biomass and PHB accumulation of 22.9 g l^{-1} and 12.6 g l^{-1} were obtained in 83 h leading to productivity of 0.15 g l^{-1} h^{-1}. But in autotrophic fermentations there was a possibility of spontaneous detonation. To prevent this oxygen concentration in gas phase had to be kept below 6.9% (v/v), which resulted in very low oxygen transfer rate.

It has been observed that N/C ratio has a significant (limiting and inhibiting) effect on overall growth and PHB accumulation in *W. eutropha*, which eventually affects dR/dt term (active biomass rate) in the model equation dR/dt also appears in RP, RSN and RSF (rates of the model equation) indicating that product formation, fructose consumption and nitrogen consumption are dependent on dR/dt. The term incorporating N/C ratio is also carried forward in the fed-batch model equations when batch model is extrapolated by adding the dilution terms. When nitrogen or fructose (or both) feeding is done in fed-batch conditions the N/C plays an important (limiting or inhibitory) effect on specific growth rate (and also on dR/dt) and the effect is passed on to other substrates (fructose and nitrogen) and product through dR/dt, making the model more realistic particularly for any inhibition due to over addition of nitrogen or carbon. Thus, N/C concentration can be controlled near optimum values by model based cultivation only.

Hence, in the present study, a different approach was used to optimize the feeding rates of limiting nutrients. The proposed batch model was extrapolated to fed-batch by incorporating dilution terms arising due to feeding of ammonium sulphate and fructose. Computer simulations of fed-batch model were used to predict the feeding strategies and feeding rates of fructose and ammonium sulphate.

Constant feeding strategy for nitrogen and fructose gave productivity of 0.11 g l^{-1} h^{-1} and a PHB production rate of 0.56 g h^{-1} which was a considerable improvement over the values obtained in batch cultivation (productivity of 0.09 g l^{-1} h^{-1}, 0.4 g h^{-1} of PHB production rate). These values are comparable to those obtained by Sugimoto et al. (1999) for autotrophic cultivation.

4. Conclusions

Batch kinetics of the PHB synthesis in a 7 l bioreactor under controlled conditions of pH and dissolved oxygen gave a total biomass of 14 g l^{-1} with a PHB content of 43.5%. The kinetic data was used for proposing a mathematical model. The model successfully simulated the observed batch kinetics. The developed mathematical model was extrapolated to fed-batch cultivation and various fed-batch strategies were simulated. Two fed-batch strategies (constant feeding of nitrogen and constant feeding of both nitrogen and fructose) were experimentally implemented. There was a considerable improvement in the biomass production rate reaching to a value of 1.54 g h^{-1} in fed-batch cultivation in which feeding of both nitrogen and fructose was carried out. The PHB content of fed-batch strategy with nitrogen feeding was only 23%. This was expected also as the limitation of nitrogen was removed due to nitrogen feeding and absence of fructose feeding led to a lesser amount of both biomass (13 g l^{-1}) and PHB (3.4 g l^{-1}). This condition was immediately improved in the next strategy where feeding of both nitrogen and fructose ensured a higher biomass (22.6 g l^{-1}), PHB concentration (8.2 g l^{-1}) and PHB content (34%).

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