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Fed-batch Optimization of PHB Synthesis through Mechanistic, Cybernetic and Neural Approaches

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***Abstract**: Despite its superiority over chemically synthesized petroleum-based polymers, poly-β-hydroxybutyrate (PHB) has been less successful commercially. A prime reason is the low productivity of microbial processes for PHB. High fermentation efficiency requires good modelling and optimization. Neither classical mechanistic models nor the recent cybernetic models have resulted in sufficiently high yields of PHB. So a neural network description has been proposed here. Relative to the other two approaches, neural optimization doubled the maximum PHB concentration in fed-batch fermentation with Ralstonia eutropha, the most commonly employed organism for PHB production, and it consumed less of the substrates. This advantage and their model-free nature make neural networks an attractive technique to enhance PHB productivity.*

***Keywords:** PHB, Fed-batch fermentation, Neural kinetics, Optimum dispersion.*

Introduction

Poly-β-hydroxybutyrate (PHB) is a microbially synthesized polymer with properties similar to those of polymers such as polyethylene and polypropylene, which are synthesized chemically from petroleum products [10, 23]. In addition, it can be biodegraded easily, whereas synthetic polymers are recalcitrant, it is compatible with body tissues, and it can be manufactured from renewable natural resources. With so many useful features, it may not be surprising that the possible applications of PHB cover a wide range of products such as biodegradable carriers for medicines and insecticides, food packaging films, disposable cosmetic products, surgical sutures and wound dressings [16, 23].

The rising costs of crude oil and of environmental degradation have led to a resurgence of interest in microbial processes for PHB. Presently, the cost of microbial PHB is four to five times that of chemically obtained polyesters [6, 40]. Since the raw materials for PHB are less expensive than those for polyethylene and polypropylene, and the production conditions are milder, the greater cost is largely due to low productivity. Therefore, improvement of fermentation efficiency is a key factor in making a microbial PHB process viable [19].

Ralstonia eutropha and *Alcaligenes latus* are commonly used bacteria for PHB production, largely because they can accumulate large amounts of PHB within the cells. While there is growing interest in *A. latus*, due to its high specific rate of PHB synthesis and the possibility of continuous fermentation, current studies are focused on *R. eutropha*, whose PHB potential can theoretically reach 80% of dry cell mass [23, 40].

The production of PHB by *R. eutropha* depends critically on the rates of supply of carbon and nitrogen. There should be sufficient, but not excess, of carbon to promote cell growth, and a

reasonable shortage, but not cessation, of nitrogen to initiate PHB synthesis [19, 44]. This suggests fed-batch fermentation as the preferred mode of operation, with the flow rates of the two feed streams being varied as the fermentation progresses [6, 19, 40]. Since optimal control of the feed rates depends on a good fermentation model and an efficient control algorithm, most studies to increase fermentation efficiency have focused on these two features.

Fermentation models in the literature are broadly of two kinds. One type are the so-called mechanistic models. They are constructed on the basis of postulated mechanisms in a manner similar to those of chemical kinetics. The models of Mulchandani et al. [28], Kim et al. [20] and Lee et al. [24] are of this type. The other kind of models is called cybernetic. This approach takes the view that living cells interact with their environment both mechanistically and through internal regulatory processes. The latter enable them to respond to changes in their surroundings by remembering their past history and in a manner that maximizes their chances of survival. This survival is generally measured by the specific growth rate, but other measures are also possible [37, 41].

Cybernetic modelling is more recent than mechanistic modelling, so there are fewer cybernetic models for PHB [13, 46].

Both cybernetic and mechanistic modelling are equation-based, i.e. they construct a set or sets of equations on the basis of certain postulates. However, in the nonideal realistic conditions of large bioreactor operations it is not easy to formulate a set of equations or estimate all the parameters or have adequate measurements for all the variables. Then a model-free method becomes useful. Neural networks offer such a method. Many fermentation studies, under both ideal and nonideal conditions, attest the usefulness of neural modelling (see the reviews by Montague and Morris [27] and Patnaik [29]).

The effectiveness of neural networks for many fermentations [26, 29, 32] suggests a similar possibility for PHB synthesis. Since the use of neural networks will add one more class of models, it is useful to know how they compare with mechanistic and cybernetic models. This comparison is important because the choice of a good modelling approach is critical in deciding the time-dependent feed rates and therefore the productivity of the fermentation.

Description of the fermentation

PHB is an energy storage polymer which the cells synthesize under adverse conditions, particularly the absence of nitrogen or phosphorus or sulfur. Of these, starvation of nitrogen is the most common method to induce polymer synthesis because (a) its supply is easy to regulate and (b) more of nitrogen than either sulfur or phosphorus is utilized and hence a deficiency of nitrogen creates greater environmental stress and consequently more PHB formation. The mechanisms underlying this process are described elsewhere [6, 23, 40].

Apart from depriving the cells of nitrogen, there should also be an adequate supply of a carbon substrate to promote growth. However, experimental studies [18, 20, 24, 44] indicate that too much of the carbon source or too little of the nitrogen source can be detrimental. The optimal ratio of carbon:nitrogen concentrations appears to be between 10 and 20. Given the complex metabolic network [6, 40], this optimum ratio is not constant but varies as the fermentation progresses and the distributions of cell ages and the concentrations of metabolites change. Studies have shown [24, 39, 42] that not only the feed rates of the nitrogen and carbon substrates but also their ratio vary nonlinearly with time.

R. eutropha catabolizes carbohydrates via the Entner-Doudorhoff pathway to pyruvate, which can then be dehydrogenated to acetyl-CoA. During reproductive growth, acetyl-CoA enters the TCA cycle and is completely oxidized to CO_2, thereby generating ATP, NADH, NADPH and biosynthetic precursors [36]. The precursors are converted to amino-acids, which are eventually incorporated into nascent proteins. The inflow of acetyl-CoA into the TCA cycle depends on the availability of nitrogen, phosphorus, sulfur and other elements. So, starving the cells of these elements, principally nitrogen, inhibits the synthesis of these proteins and leads to high accumulations of NADH and NADPH. These in turn slow down the TCA cycle and channelize acetyl-CoA toward PHB synthesis [8].

While nitrogen starvation may generate high intra-cellular concentrations of PHB, it also slows cell growth and thus lowers the accumulations of PHB in the broth [20, 24]. So, an initially high cell density, achieved through balanced growth, is required before polymer synthesis is triggered. Thereafter, the relative flow rates of carbon and nitrogen have to be manipulated in response to cell growth and product formation. The reported time-variant nature of the two feed rates lends itself more easily to fed-batch fermentation, which therefore produces greater concentrations of PHB than continuous fermentation [6, 23, 38]. The carbon source is either fructose or glucose, and nitrogen is provided by a solution of either ammonium chloride or ammonium sulfate.

Kinetic and reactor modelling approaches

Bioreactor model

Most of the published studies of PHB fermentation have focused on small, laboratory-scale bioreactors, typically 2 to 5 l in volume. In such vessels, mixing is usually sufficiently good to avoid practically significant spatial gradients of the concentrations of interest. For such fed-batch fermentations, the reactor model for arbitrary kinetics may be expressed by the mass balances given below [2].

$$\frac{dx}{dt} = r_X - \frac{x}{V}\frac{dV}{dt} \tag{1}$$

$$\frac{dp}{dt} = r_p - \frac{p}{V}\frac{dV}{dt} \tag{2}$$

$$\frac{ds_C}{dt} = r_C + \frac{s_{Cf} F_C}{V} - \frac{s_C}{V}\frac{dV}{dt} \tag{3}$$

$$\frac{ds_N}{dt} = r_N + \frac{s_{Nf} F_N}{V} - \frac{s_N}{V}\frac{dV}{dt} \tag{4}$$

$$\frac{dV}{dt} = F_C(t) + F_N(t) \tag{5}$$

The initial values of x, p, s_C and s_N are known, and the best starting volume was determined as part of the optimization process.

Equations (1) - (5) assume that fluid dispersion in the fermentation broth is complete and therefore there are no spatial gradients. This assumption is, however, not valid for large bioreactors. Experimental measurements with reactors of different sizes and by using different techniques [4, 15, 26, 35] have shown that substrate and dissolved oxygen gradients can be substantial even in pilot-scale vessels. While gradients may be reduced by better agitation, they cannot be eliminated totally from large (production scale) bioreactors. The conventional

a priori expectation is that gradients have a negative effect on bioreactor performance. However, recent studies [3, 14, 15, 30] have shown that controlled dispersion, i.e. limited gradients, can be beneficial. Indeed, there appears to be an optimal level of dispersion for a given fermentation system [30, 33, 34] that generates a better performance than complete dispersion or too little dispersion.

The degree of dispersion may be quantified by the Peclet number, defined as:

$$Pe = uL / D_e \tag{6}$$

When the fluid is fully dispersed, $D_e \to \infty$ and hence $Pe \to 0$. At the other extreme is fully segregated flow, for which $D_e \to 0$ and $Pe \to \infty$. In small bioreactors, Pe can be small, whereas it has finite non-zero values in large reactors. When there is finite dispersion, Eqs. (1) - (4) are modified to [2, 12]:

$$\frac{V}{F}\frac{\partial x}{\partial t} = \frac{\partial^2 x}{\partial z^2} - Pe\frac{\partial x}{\partial z} + \frac{V}{F}r_X \tag{7}$$

$$\frac{V}{F}\frac{\partial p}{\partial t} = \frac{\partial^2 p}{\partial z^2} - Pe\frac{\partial p}{\partial z} + \frac{V}{F}r_P \tag{8}$$

$$\frac{V}{F}\frac{\partial s_C}{\partial t} = \frac{\partial^2 s_C}{\partial z^2} - Pe\frac{\partial s_C}{\partial z} - \frac{V}{F}r_C + \frac{F_C}{F}s_{Cf} \tag{9}$$

$$\frac{V}{F}\frac{\partial s_N}{\partial t} = \frac{\partial^2 s_N}{\partial z^2} - Pe\frac{\partial s_N}{\partial z} - \frac{V}{F}r_N + \frac{F_N}{F}s_{Nf} \tag{10}$$

where $F = F_C + F_N$ = total inflow rate of the carbon and nitrogen sources.

Suitable expressions are used for the kinetic rates r_C, r_N, r_P and r_X. These may be derived through either a mechanistic approach or a cybernetic approach. Both methods have been applied here, and the results compared with a new model-free approach utilizing artificial neural networks.

Kinetic models

The classical approach is to derive kinetic equations on the basis of postulated reaction networks, just as for homogeneous chemical reactions. In this class, the mechanistic model of Lee et al. [24] was chosen because (a) it is simple and physiologically meaningful, (b) it has yielded good results and (c) it is based on *R. eutropha* NCIMB 11599, for which a good cybernetic model [46] is available for comparison. The detailed equations are given in Appendix A.

A major weakness of mechanistic kinetics is its assumption that living cells respond to environmental changes in the same manner as chemical species. Observations of microbial culture dynamics do not support this assumption. There are many examples (discussed by Patnaik [31]) which show that mechanistic models either fail to predict or do not predict satisfactorily fundamental features such as the initial lag phase and transient responses to perturbations.

These limitations also showed up in Yoo and Kim's [46] study of PHB kinetics in batch fermentations. So they proposed an alternate cybernetic model based on concepts introduced by Ramkrishna and coworkers. Briefly, the cybernetic approach takes the view that microbial behavior is regulated both by the environment and by internal regulatory processes. As a

result, the cells adjust their metabolic processes so as to maximize their chances of survival under the prevailing conditions [9, 31].

In Yoo and Kim's [46] cybernetic model, "the carbon source is optimally allocated to the key enzyme synthesis system so that the cells have a high degree of flexibility under nitrogen starvation". Under the stress created by a shortage of nitrogen, the cells direct the remaining resources toward the synthesis of PHB [6, 40]. Like Lee et al. [24], Yoo and Kim [46] also divided each cell into a PHB component and residual biomass. Detailed equations are provided in Appendix B.

In production and pilot scale bioreactors, fluid mixing is usually incomplete and may vary as the fermentation progresses. In addition, continuous and fed-batch operations are often subject to 'noise' carried by inflow streams. Under such conditions, microbial behavior is complex, time-dependent and difficult to monitor and predict accurately [3, 4, 25]. Then the mathematical models described above are inadequate, and a sufficiently good model becomes extremely complex and thus difficult to use in on-line applications.

By not requiring a mathematical description of the fermentation, arrays of artificial neural networks circumvent these limitations. Previous work [7, 32] has shown that a recurrent neural network is more appropriate for an incompletely mixed bioreactor than other common configurations. In a recurrent network output signals from downstream neurons are fed back to a layer of upstream neurons, and this feature mimics the internal recirculation in a broth with finite dispersion. Such a network has two basic variants. In the Elman form, outputs from the hidden neurons are recycled to the input layer, while in the Jordan form the signals from the output layer are recycled to the input layer. Because the circulation of fluid occurs inside the broth, the Elman form represents internal mixing more faithfully than the Jordan network [32].

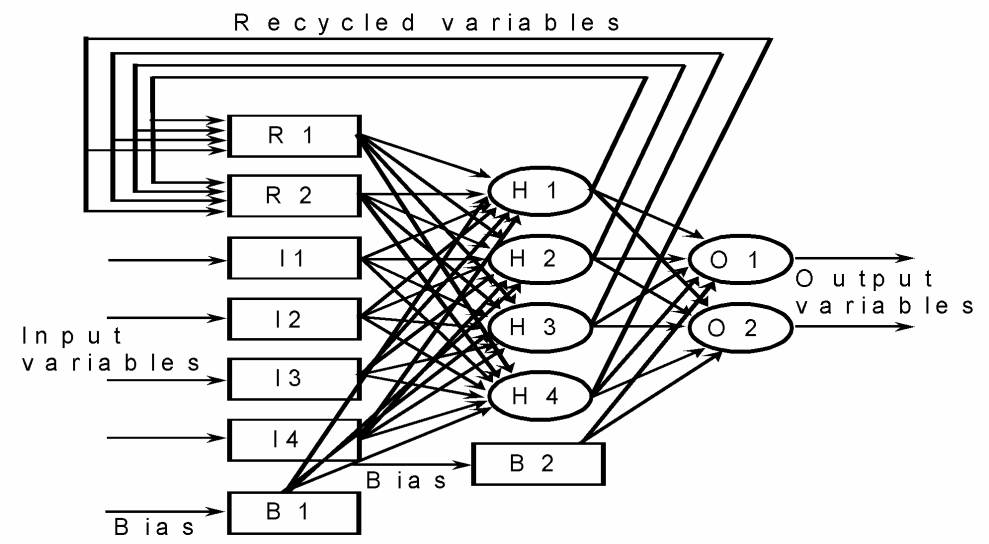

Fig. 1 Configuration of the Elman neural network for PHB kinetics

For the present application, the Elman network had the configuration shown in Fig. 1. There are four input neurons to receive data of the concentrations and flow rates of the nitrogen and carbon substrates. Similarly, the residual (or total) biomass and PHB are the main outputs of interest. So the concentrations of these two variables are expressed by the output neurons. In a

feedback control system, these two concentrations are used to manipulate the flow rates of the two substrates. This is mimicked in an Elman neural network by recycling the output concentrations into the input layer. Then, two more neurons are required to receive these signals; these are shown in Fig. 1 as the recurrent neurons R1 and R2. In addition, there are two bias neurons B1 and B2. These are optional mathematical devices to help convergence and avoid the optimization process from getting trapped in a trough [17]. While the inputs and outputs fix the number of neurons in those layers, the number in the hidden layer was varied during training of the network with data from Lee et al. [24] and Yoo and Kim [46]. The optimum number of hidden neurons was determined to be four.

Results and discussion

To compare the modelling approaches under both ideal and nonideal conditions, they were applied to the fermentation at complete dispersion and at optimum dispersion in the bioreactor. Complete dispersion is characterized by Pe = 0. In a recent publication [34] it has been shown that Pe = 20 maximizes the concentration of PHB. The reasons for the best performance at a finite dispersion are discussed in that article and are briefly considered here.

The performances at these two values of Pe and for the three kinds of models are portrayed in Figs. 2 - 5. Each figure has three pairs of plots, one pair each for the mechanistic model, the cybernetic model and the neural model. For the two output variables, the residual biomass (Fig. 2) and PHB (Fig. 3), the lower plot in each pair corresponds to Pe = 0 and the upper plot to Pe = 20. This situation is reversed for the two input variables, i.e. the carbon source (Fig. 4) and the nitrogen source (Fig. 5). This difference provides a graphic demonstration of the benefit of optimal dispersion, irrespective of the type of kinetic model used. At Pe = 20 there is better growth of cells, greater synthesis of PHB and improved utilization of both glucose and ammonium chloride than achieved in a fully dispersed (ideal) bioreactor.

With a mechanistic model, the concentration of residual biomass (Fig. 2) saturates in about 20 h and that of PHB (Fig. 3) increases slowly with time. (Hence the sum of the two, i.e. the total biomass, also increases slowly with time). On the contrary, both concentrations increase faster with cybernetic and neural kinetics, the neural model generating consistently higher concentrations. This improvement in bioreactor performance is also reflected in the consumption of nutrients, where the unutilized glucose and ammonium chloride are the least with an optimized neural model and the highest with a mechanistic model (Figs. 4 and 5).

These results are summarized quantitatively in Tables 1 and 2, which display the final percentage differences between different kinetic approaches and between complete dispersion (Pe = 0) and optimum dispersion (Pe = 20). While there are consistent improvements from a mechanistic to a cybernetic to a neural model and from Pe = 0 to Pe = 20, more significant perhaps are their magnitudes, which range from 14.6% to 129.5%. For both values of the Peclet number, the increases in PHB content are always larger than the corresponding increases in residual biomass. Thus, a change in the kinetic formalism alone enables better optimization of the fermentation, regardless of the degree of dispersion, thereby enhancing PHB yields per unit volume of the broth and per unit mass of cells. Moreover, these improvements are achieved with reduced consumption of glucose and ammonium chloride.

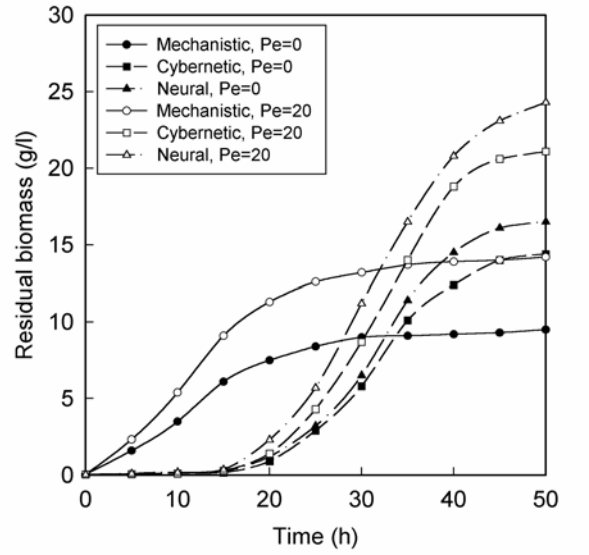

Fig. 2 Profiles of the residual biomass in the bioreactor predicted by different kinetic models. For each pair of plots, the one with filled symbols is for Pe = 0 and the plot with open symbols is for Pe = 20.

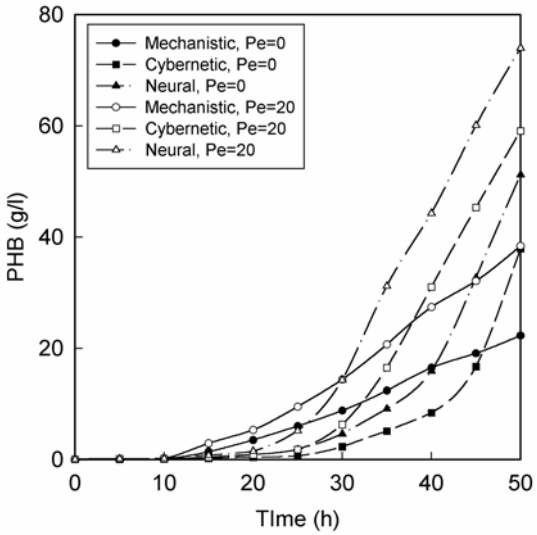

Fig. 3 Profiles of PHB concentration in the bioreactor predicted by different kinetic models. For each pair of plots, the one with filled symbols is for Pe = 0 and the plot with open symbols is for Pe = 20.

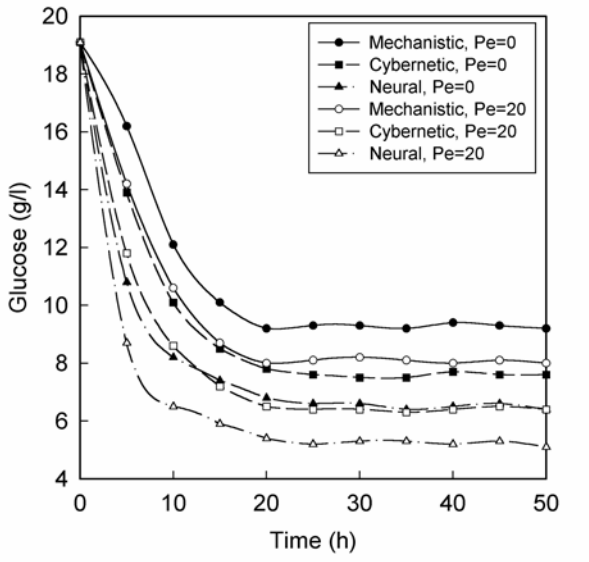

Fig. 4 Profiles of glucose concentration in the bioreactor predicted by different kinetic models. For each pair of plots, the one with filled symbols is for Pe = 0 and the plot with open symbols is for Pe = 20.

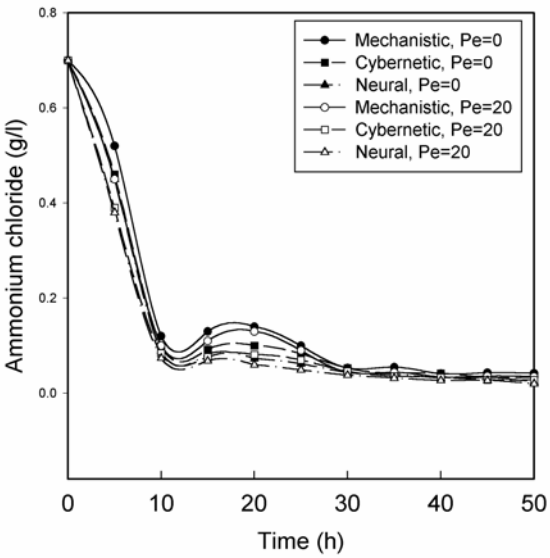

Fig. 5 Profiles of ammonium chloride concentration in the bioreactor predicted by different kinetic models. For each pair of plots, the one with filled symbols is for Pe = 0 and the plot with open symbols is for Pe = 20.

Table 1. Percentage increases or reductions in the final concentrations of different variables at the end of the fermentation

Concentration variable	Between different kinetic models					
	Mechanical→Cybernetic		Cybernetic→Neural		Mechanical→Neural	
	Pe = 0	Pe = 20	Pe = 0	Pe = 20	Pe = 0	Pe = 20
Residual biomass	51.6	48.6	14.6	15.2	73.7	71.1
PHB	69.8	53.3	35.4	24.4	129.5	92.9
Glucose*	-21.0	-20.0	-15.8	-20.3	-29.3	-36.2
Ammonium Chloride*	-19.0	-20.0	-29.4	-28.6	-42.8	-42.8

Table 2. Percentage increases or reductions in the final concentrations of different variables at the end of the fermentation

Concentration variable	From Pe = 0 to Pe = 20		
	Mechanical	Cybernetic	Neural
Residual biomass	49.5	46.5	47.3
PHB	72.1	55.8	54.6
Glucose*	-13.0	-15.8	-20.3
Ammonium Chloride*	-16.7	-17.6	-16.8

* Negative values for these concentrations mean reductions.

The superior performance with a neural model continues for the feed rates of glucose and ammonium chloride. The plots for glucose (Fig. 6) show that for a given model, less of the substrate is required at the optimum dispersion (Pe = 20) than for complete dispersion (Pe = 0). Similarly, for a given degree of dispersion, neural kinetics ensures less of glucose requirement than cybernetic and mechanistic kinetics. Corresponding plots for the nitrogen source (not shown) had the same trends.

While it is possible, on the basis of these results, to recommend neural kinetics for fed-batch fermentation for PHB synthesis, it is also useful to seek explanations for the observations. There are two important issues. First, why are neural models better than cybernetic models, and mechanistic models the least efficient? In answer to this, we note that a cybernetic model portrays experimental data more accurately than a mechanistic model. This has been attributed [9, 21, 22, 46] to the inclusion of intra-cellular regulatory controls and of biological 'memory' in cybernetic models, which are absent in mechanistic models.

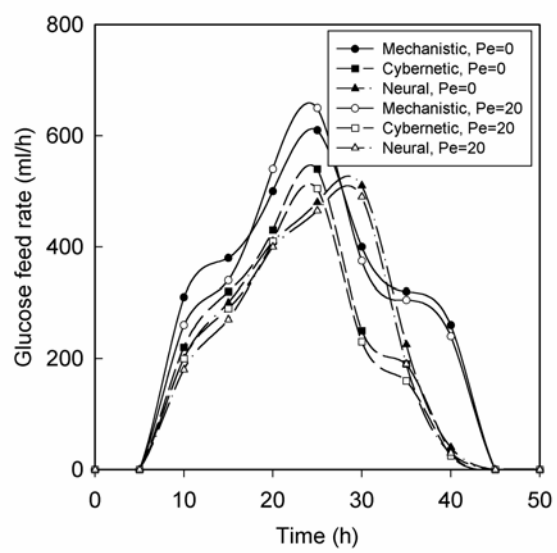

Fig. 6 Variations of the optimal glucose feed rate predicted by different kinetic models as the fermentation progresses. For each pair of plots, the one with filled symbols is for Pe = 0 and the plot with open symbols is for Pe = 20.

However, cybernetic models have other limitations. One is that the models can sometimes become prohibitively complex. Secondly, it is often difficult to draw correspondences between the key enzymes in a kinetic model and the enzymes actually present in a metabolic network. Until now, such identifications have been proposed heuristically such that model predictions agree with experimental results [41, 43, 46]. A third difficulty is that more than one cybernetic objective (i.e. maximization of different goals) can be proposed for a given set of observations, and there is yet no rationale to rule out a (weighted) combination of objectives.

Both cybernetic and mechanistic models have a common limitation when applied to large nonideal bioreactors. Because the conditions in the broth change with both time and location, these models have limited ability and flexibility to predict such variations. Neural networks do not have these weaknesses, mainly because (a) they do not require a mathematical model, (b) they are trained with actual large-scale data, (c) they learn and improve with usage, and (d) they are robust to disturbances and measurement errors [27, 29]. These features help a neural network to maintain a consistently close fidelity to the fermentation process and consequently optimize it to generate higher outputs than cybernetic and mechanistic models.

The second issue concerns the existence of an optimal dispersion, which goes contrary to conventional bioreactor theory. In a fully dispersed broth, the nutrients, products and intermediates are uniformly distributed. Now, the mechanism of PHB synthesis is such that its formation is favored by sufficient amounts of carbon and a shortage of nitrogen [6, 19, 40]. However, a preponderance of carbon over nitrogen is not desirable either, and the optimal ratio varies between 10 and 20. Now, while nitrogen starvation promotes PHB synthesis, this is also coupled with degradation of the polymer. Doi et al.'s [11] experiments showed that under restricted nitrogen supply the synthesis of PHB and its degradation to a PHB-co-PHV (PHV = poly-hydroxyvalerate) copolymer formed a cyclic process.

The formation of PHB is also connected with the formation and consumption of acetate, an intermediate. High concentrations of PHB suppress acetate formation; this is desirable since

acetate inhibits cell growth. However, extremely fast synthesis of PHB is also unfavorable to the cells since it exerts a high metabolic stress [45]. Therefore, the production of PHB and of acetate have to balanced to optimize the fermentation [5].

The degree of mixing or dispersion in the broth plays an important role in determining the balances between synthesis and degradation of PHB and between PHB production and acetate production. At complete dispersion the carbon and nitrogen substrates are available freely at all positions in the broth. This favors acetate formation, thus inhibiting cell growth and resulting in low volumetric productivity of PHB, even though its intra-cellular concentration may be high [6, 40, 45]. On the other hand, poor dispersion restricts the availability of the substrates to the cells, and hence there is low synthesis of PHB. Between these two extreme situations there is a finite degree of dispersion that maximizes PHB production without excessive acetate formation. In fact, the role of acetate is more complicated than the short account given above. The concentration of acetate in the bioreactor depends not only on that of PHB but also on glucose concentration and therefore indirectly on the C:N ratio. At low glucose concentrations, acetate provides a supplementary source of carbon so that this ratio does not decrease too much. Thus, acetate is involved in a rather complex way in the metabolism of carbon and nitrogen, and this is manifested on a macroscopic level by the undulating plots of ammonium chloride (Fig. 5). These variations are less conspicuous for glucose since its concentration is much higher than that of ammonium chloride.

These considerations suggest that the carbon and nitrogen sources should neither be freely available everywhere in the both (as with Pe = 0) nor confined to large stagnant regions with little access to the cells (when Pe is very large). An optimal balance between PHB formation and degradation, on the one hand, and between PHB synthesis and the net rate of acetate formation, on the other, requires an intermediate degree of dispersion. For PHB synthesis by *R. eutropha*, this is attained at Pe = 20. For both this level of dispersion and at complete dispersion, optimization of the fermentation through a neural representation generated a greater concentration of PHB and required less substrate consumption than with a mechanistic model or a cybernetic model. This observation is useful for large bioreactors because, as explained earlier, it is difficult to formulate models that are simple, flexible and sufficiently accurate.

Conclusions

In terms of its useful properties, PHB compares favorably with synthetic polymers such as polypropylene. However, owing to its low productivity, the microbial production of PHB has not yet become competitive with the chemical synthesis of petroleum-based polymers.

Increase of productivity requires a good quantitative description and proper optimization of the fermentation. Three kinetic modelling approaches – mechanistic, cybernetic and neural – have been compared for a fed-batch fermentation using *Ralstonia eutropha*. Each model was applied to a fully dispersed bioreactor (Pe = 0) and one operated at optimum dispersion (Pe = 20). For either value of Pe, both cell growth and PHB synthesis were highest with a neural kinetic model and lowest with a mechanistic model. The performance at Pe = 20 was superior to that at Pe = 0 for all three models. Moreover, for both complete dispersion and optimum dispersion, the increases in PHB concentration upon shifting from a mechanistic model to a cybernetic model or to a neural model were larger than those of the residual biomass.

For a fully dispersed bioreactor, a neural network for the kinetics generated 130% more PHB at the end of the fermentation than with a mechanistic model. No less significant was the improvement of 93% at optimum dispersion. These improvements were accompanied by reduced consumption of glucose and ammonium chloride, the two main substrates. Since large bioreactors have incomplete dispersion and are difficult to model, the results thus suggest that neural networks may be utilized to exploit the finite dispersion to increase the yields in the microbial synthesis of PHB to commercially viable levels.

Nomenclature

D_e effective dispersion coefficient, [$cm^2 \cdot h^{-1}$]
E_i specific activity of i-th key enzyme, [-]
F_C feed rate of carbon source, [$l \cdot h^{-1}$]
F_N feed rate of nitrogen source, [$l \cdot h^{-1}$]
K_C Monod constant for growth on carbon source, [$g \cdot l^{-1}$]
K_{CI} inhibition constant for growth on carbon source, [$g \cdot l^{-1}$]
K_N Monod constant for growth on nitrogen source, [$g \cdot l^{-1}$]
K_{NI} inhibition constant for growth on nitrogen source, [$g \cdot l^{-1}$]
K_{PC} Monod constant for production of PHB on carbon source, [$g \cdot l^{-1}$]
K_{PCI} inhibition constant for production of PHB on carbon source, [$g \cdot l^{-1}$]
K_{PN} Monod constant for production of PHB on nitrogen source, [$g \cdot l^{-1}$]
K_{PNI} inhibition constant for production of PHB on nitrogen source, [$g \cdot l^{-1}$]
L characteristic dimension of bioreactor, [cm]
m_e specific maintenance energy, [$l \cdot h^{-1}$]
p product (PHB) concentration, [$g \cdot l^{-1}$]
Pe Peclet number, [-]
r_C rate of consumption of carbon source, [$g \cdot l^{-1} \cdot h^{-1}$]
r_N rate of consumption of nitrogen source, [$g \cdot l^{-1} \cdot h^{-1}$]
r_P rate of formation of product, PHB, [$g \cdot l^{-1} \cdot h^{-1}$]
r_X rate of formation of biomass, [g/l/h]
s_C carbon source concentration, [$g \cdot l^{-1}$]
s_{Cf} carbon source concentration in feed stream, [$g \cdot l^{-1}$]
s_N nitrogen source concentration, [$g \cdot l^{-1}$]
s_{Nf} nitrogen source concentration in feed stream, [$g \cdot l^{-1}$]
t real time, [h]
u characteristic fluid in bioreactor, [$cm \cdot h^{-1}$]
v_i fractional allocation of substrate i to cellular processes, [-]
V volume of broth in the bioreactor, [l]
V_w working volume of bioreactor, [l]
x total biomass concentration, [$g \cdot l^{-1}$]
x_r residual biomass concentration, [$g \cdot l^{-1}$]
$Y_{R/C}$ yield coefficient for residual biomass on carbon source, [$g \cdot g^{-1}$]
$Y_{P/C}$ yield coefficient for PHB on carbon source, [$g \cdot g^{-1}$]
$Y_{R/N}$ yield coefficient for residual biomass on nitrogen source, [$g \cdot g^{-1}$]
α_i synthesis rate constant for i-th key enzyme, [$l \cdot h^{-1}$]
β_i decay rate constant for i-th key enzyme, [$l \cdot h^{-1}$]
γ_i cybernetic variable for substrate i, [-]
μ specific growth rate of residual biomass, [$l \cdot h^{-1}$]
π specific rate of formation of PHB, [$l \cdot h^{-1}$]
σ_C specific consumption rate of carbon source, [$l \cdot h^{-1}$]
σ_N specific consumption rate of nitrogen source, [$l \cdot h^{-1}$]

Appendix A. The mechanistic model of Lee et al. [24]

Lee et al. [24] used a small (2.5 l) bioreactor, in which spatial gradients could be ignored. Their fed-batch model was:

$$\frac{dx_r}{dt} = \mu x_r; \qquad x_r(0) = x_{r0} \tag{A1}$$

$$\frac{ds_C}{dt} = s_{Cf} F_C - \sigma_N x_r; \qquad s_C(0) = s_{C0} \tag{A2}$$

$$\frac{ds_N}{dt} = s_{Nf} F_N - \sigma_N x_r; \qquad s_N(0) = s_{N0} \tag{A3}$$

$$\frac{dp}{dt} = \pi x_r; \qquad p(0) = p_0 \tag{A4}$$

$$\frac{dV}{dt} = F_C + F_N; \qquad V(0) = V_0 \tag{A5}$$

The working volume, V_w, of the bioreactor sets the upper limit on V. The process is stopped at or before $V = V_w$.

For the specific rates μ, σ_C and σ_N, Lee et al. [24] modified Asenjo and Suk's [1] product inhibition model to include the production of PHB without ammonium.

$$\mu = \mu_m \left(\frac{s_C}{K_C + s_C + s_C^2 / K_{CI}} \right) \left(\frac{s_N}{K_N + s_N + s_N^2 / K_{NI}} \right) \tag{A6}$$

$$\pi = \pi_m \left(1 - \frac{p / x_r}{(p / x_r)_m} \right) \left(\frac{s_C}{K_{PC} + s_C + s_C^2 / K_{PCI}} \right) \left(\frac{s_N + K_P}{K_{PN} + s_N + s_N^2 / K_{PNI}} \right) \tag{A7}$$

$$\sigma_C = \left(\frac{\mu}{Y_{R/C}} + \frac{\mu}{Y_{P/C}} + m_e \right) \tag{A8}$$

$$\sigma_N = \mu / Y_{R/N} \tag{A9}$$

The values of the parameters are listed in Table 3.

Appendix B. The cybernetic model of Yoo and Kim [46]

Yoo and Kim [46] expressed the growth rate of residual biomass by modified Monod kinetics as:

$$r_X = \frac{dx_r}{dt} = \frac{\mu E_1 s_N x_r}{K_N + s_N} \tag{B1}$$

A similar expression was formulated for the rate of PHB synthesis:

$$r_P = \frac{\pi E_2 s_C x_r}{K_C + s_C} \tag{B2}$$

Yoo and Kim [46] interpreted maximization of the cells' survival as maximization of the cell mass at each instant of time. So at any time the cells allocate each resource, i.e. glucose or ammonium salt, such that their fractional allocations follow:

$$v_i = \frac{\exp(r_i)}{\exp(\sum_j r_j)};\ i, j = C \text{ or } N \quad \text{(B3)}$$

The activity of the key enzyme E_i for cell growth or PHB synthesis on substrate i was expressed as:

$$\gamma_i = \frac{\exp(r_i)}{\max_j[\exp(r_j)]};\ i, j = C \text{ or } N \quad \text{(B4)}$$

The γ_i are the cybernetic variables. If γ_i decreases, the corresponding growth and synthesis processes become slower, even with sufficient amount of the enzyme E_i.

The rate of change of each key enzyme depends on its induced synthesis, its degradation and its dilution due to cell growth.

$$\frac{dE_i}{dt} = \frac{\alpha_i s_i v_i}{K_i + s_i} - E_i v_j \left(\beta_i + \frac{d \ln x}{dt}\right);\ i, j = C \text{ or } N \quad \text{(B5)}$$

The cybernetic variables control the rates of nitrogen and carbon utilization, which have familiar forms [21].

$$-\frac{ds_N}{dt} = \frac{\mu_N r_N}{Y_N} v_N \quad \text{(B6)}$$

$$-\frac{ds_C}{dt} = \frac{\mu_N r_N}{Y_N'} v_N + \frac{\mu_C r_C}{Y_C} v_C + m_e x_r \quad \text{(B7)}$$

The growth rate of the total biomass is the sum of its rates on the individual substrates.

$$\frac{dx}{dt} = r_C + r_N \quad \text{(B8)}$$

Since PHB is accumulated intracellularly,

$$x = x_r + p \quad \text{(B9)}$$

Following Kompala et al. [32], they expressed the maximum specific growth rate on the i-th substrate as:

$$\mu_i = \frac{\mu_{mi}(\mu_{mi} + \beta_i)}{\alpha_i} \quad \text{(B10)}$$

Here i is either glucose (C) or ammonium chloride (N). Table 4 lists the values of the parameters from Yoo and Kim [46].

Table 3. Values of the parameters in the mechanistic model of Lee et al. [10]

Parameter	Units	Value
K_C	$g \cdot l^{-1}$	5.81
K_{CI}	$g \cdot l^{-1}$	14.5
K_N	$g \cdot l^{-1}$	0.69
K_{NI}	$g \cdot l^{-1}$	0.15
K_P	$g \cdot l^{-1}$	0.05
K_{PC}	$g \cdot l^{-1}$	2.09
K_{PCI}	$g \cdot l^{-1}$	80.0
K_{PN}	$g \cdot l^{-1}$	0.05
K_{PNI}	$g \cdot l^{-1}$	0.9
m_e	$1 \cdot h^{-1}$	0.01 (0 when $s_C = 0$)
$(P/X)_m$	-	0.85
$Y_{P/C}$	$g \cdot g^{-1}$	0.47
$Y_{R/C}$	$g \cdot g^{-1}$	0.45
$Y_{R/N}$	$g \cdot g^{-1}$	2.11
μ_m	$1 \cdot h^{-1}$	0.875
π_m	$1 \cdot h^{-1}$	0.402

Table 4. Values of the parameters in the cybernetic model of Yoo and Kim [46]

Parameter	Units	Value
K_N	$g \cdot l^{-1}$	0.254
K_C	$g \cdot l^{-1}$	3.804
$(P/X)_0$	-	0.569
Y_N	$g \cdot g^{-1}$	1.653
Y'_N	$g \cdot g^{-1}$	0.460
Y_C	$g \cdot g^{-1}$	0.439
m_e	$1 \cdot h^{-1}$	0.010
α_i (i = C or N)	$1 \cdot h^{-1}$	0.001
β_i (i = C or N)	$1 \cdot h^{-1}$	0.050
μ_{mN}	$1 \cdot h^{-1}$	0.176
μ_{mC}	$1 \cdot h^{-1}$	0.098

References

1. Asenjo J. A., J. S. Suk (1985). Kinetics and Models for the Bioconversion of Methane into an Intra-cellular Polymer, Poly-β-hydroxybutyrate (PHB), Biotechnol. Bioeng. Symp., 15, 225-234.
2. Blanch H. W., D. S. Clark (1996). Biochemical Engineering, New York, Marcel Dekker, Ch. 4.
3. Bylund F., A. Castan, R. Mikkola, A. Viede, G. Larsson (2000). Influence of Scale-up on the Quality of Recombinant Human Growth Hormone, Biotechnol. Bioeng., 69, 119-128.
4. Bylund F., F. Guillard, S. E. Enfors, C. Tragardh, G. Larsson (1999). Scale Down of Recombinant Protein Production a Comparative Study of Scaling Performance, Bioproc. Eng., 20, 377-389.
5. Braunegg G., G. Lefebre, G. Renner, A. Zeiser, G. Haage, K. Loidl-Lanthaler (1995). Kinetics as a Tool for Polyhydroxyalkanote Production Optimization, Can. J. Microbiol., 41, 239-248.
6. Braunegg G., G. Lefebre, K. F. Genser (1998). Polyhydroxyalkanoates, Biopolymers from Renewable Resources: Physiological and Engineering Aspects, J. Biotechnol., 65, 127-161.
7. Chen Q., W. A. Weigand (1994). Dynamic Optimization of Nonlinear Processes by Combining Neural Network Model with UDMC, A. I. Ch. E. J., 40, 1488-1497.
8. Dawes E. A., P. J. Senior (1973). The Role and Regulation of Energy Reserve Polymers in Microorganisms, Adv. Microbiol. Physiol., 10, 135-266.
9. Dhurjati P., D. Ramkrishna, M. C. Flickinger, G. T. Tsao (1985). A Cybernetic View of Microbial Growth: Modeling of Cells as Optimal Strategists, Biotechnol. Bioeng., 27, 1-9.

10. Doi Y. (1990). Microbial Polyesters, New York, VCH.
11. Doi Y., A. Segawa, Y. Kawaguchi, M. Kunioka (1990). Cyclic nature of Poly(3-hydroxyalkanoate) Metabolism in *Alcaligenes eutrophus*, FEMS Microbiol. Lett., 67, 165-170.
12. Froment G. F., K. B. Bischoff (1990). Chemical Reactor Analysis and Design, New York, John Wiley, Ch. 11.
13. Gadkar K. G., F. J. Doyle III, T. J. Crowley, J. D. Varner (2003). Cybernetic Model Predictive Control of a Continuous Bioreactor with Cell Recycle, Biotechnol. Prog., 19, 1487-1497.
14. Ganduri V. S. R. K., S. Ghosh, P. R. Patnaik (2004). Mixing Control as a Device to Increase PHB Production in Batch Fermetnations with Co-cultures of *Lactobacillus delbrueckii* and *Ralstonia eutropha*, Process. Biochem., 40, 257-264.
15. George S., G. Larsson, S.-O. Enfors (1993). A Scale-down Two Compartment Reactor with Controlled Substrate Oscillations: Metabolic Response of *Saccharomyces cerevisiae*, Bioproc. Eng., 9, 249-257.
16. Hanley Z. Z., T. T. Salbas, K. M. Elborough (2000). The Use of Plant Biotechnology for the Production of Biodegradable Plastics, Trends Plant Sci., 5, 45-46.
17. Hassoun M. H. (1995). Fundamentals of Artificial Neural Networks, Cambridge, MIT Press, MA, 1995.
18. Katoh T., D. Yuguchi, H. Yoshii, H. Shi, K. Shimizu (1996). Dynamics and Modeling on Fermentative Production of Poly(β-hydroxybutyric Acid) from Sugars via Lactate by a Mixed Culture of *Lactobacillus delbrueckii* and *Alcaligenes eutrophus*, J. Biotechnol., 27, 113-134.
19. Khanna S., A. K. Srivastava (2005). Recent Advances in Microbial Polyhydroxyalkanoates, Process Biochem., 40, 607-619.
20. Kim B. S., S. C. Lee, S. Y. Lee, H. N. Chang, Y. K. Chang, S. I. Woo (1994). Production of Poly(3-hydroxybutyric acid) by Fed-batch Culture of *Alcaligenes eutrophus* with Glucose Concentration Control, Biotechnol. Bioeng., 43, 892-898.
21. Kompala D. S., D. Ramkrishna, G. T. Tsao (1984). Cybernetic Modeling of Microbial Growth on Multiple Substrates, Biotechnol. Bioeng., 26, 1272-1281.
22. Kompala D. S., D. Ramkrishna, N. B. Jansen, G. T. Tsao (1986). Investigation of Bacterial Cultures at Low Growth Rates: Mixed-substrate Systems, Biotechnol. Bioeng., 28, 1044-1055.
23. Lee S. Y., H. N. Chang (1995). Production of Poly(hydroxyalkanoic Acid), Adv. Biochem. Eng. Biotechnol., 52, 27-58.
24. Lee J. H., H. C. Lim, J. Hong (1997). Application of Nonsingular Transformation to On-line Optimal Control of Poly-β-hydroxybutyrate Fermentation, J. Biotechnol., 55, 135-150.
25. Lubbert A., S. B. Jorgensen (2001). Bioreactor Performance: A More Scientific Approach for Practice, J. Biotechnol., 85, 187-212.
26. Mayr B., P. Horvat, A. Moser (1992). Engineering Approach to Mixing Quantification in Bioreactors, Bioproc. Eng., 8, 137-143.
27. Montague G. A., A. J. Morris (1994). Neural Network Contributions in Biotechnology, Trends Biotechnol., 12, 312-324.
28. Mulchandani A., J. H. T. Luong, C. Groom (1989). Substrate Inhibition Kinetics for Microbial Growth and Synthesis of Poly-β-hydroxybutyric Acid, Appl. Microbiol. Biotechnol., 30, 11-17.
29. Patnaik P. R. (1998). Neural Network Applications to Fermentation Processes, In: Bioseparation and Bioprocessing, Ed. G. Subramanian, Weinheim, Wiley-VCH, Vol. I, Ch. 14.

30. Patnaik P. R. (2000). On the Improvement of Bacterial Growth on Complementary Substrates by Partial Segregation in the Broth, J. Chem. Technol. Biotechnol., 75, 229-236.
31. Patnaik P. R. (2000). Are Microbes Intelligent Beings? An Assessment of Cybernetic Modeling, Biotechnol. Adv., 18, 267-288.
32. Patnaik P. R. (2001). A Simulation Study of Neural Filtering and Control of a Fed-batch Bioreactor under Nonideal Conditions, Chem. Eng. J., 84, 533-541.
33. Patnaik P. R. (2003). Effect of Fluid Dispersion on Cybernetic Control of Microbial Growth on Substitutable Substrates, Bioproc. Biosyst. Eng., 25, 315-321.
34. Patnaik P. R. (2006). Dispersion Optimization to Enhance PHB Production in Fed-batch Cultures of *Ralstonia eutropha*, Bioresource Technol., 97, 1994-2001.
35. Pedersen A. G., M. Bundgard-Nielsen, J. Nielsen, J. Villadsen (1994). Characterization of Mixing in Stirred Bioreactors Equipped with Rushton Turbines, Biotechnol. Bioeng., 44, 1013-1017.
36. Pranamuda H., Y. Tokiwa, H. Tanaka (1995). Microbial Degradation of an Aliphatic Polyester with a High Melting Point, poly(tetramethylene succinate), Appl. Environ. Microbiol., 61, 1828-1832.
37. Ramkrishna D., D. S. Kompala, G. T. Tsao (1987). Are Microbes Optimal Strategists? Biotechnol. Prog., 3, 121-126.
38. Ramsay B. A., K. Lomaliza, C. Chavarie, B. Dube, P. Bataille, J. A. Ramsay (1990). Production of Poly-(β-hydroxybutyric-co-β-hydroxyvaleric) Acids, Appl. Environ. Microbiol., 56, 2093-2098.
39. Riascos C. A. M., J. M. Pinto (2004). Optimal Control of Bioreactors: A Simultaneous Approach for Complex Systems, Chem. Eng. J., 99, 23-34.
40. Steinbuchel A. (1996). PHB and Other Polyhydroxyalkanoic Acids, In: Biotechnology, Ed. H.-J. Rehm, G. Reed Wienheim, VCH, Vol. 6, Ch. 13.
41. Straight J. V., D. Ramkrishna (1994). Cybernetic Modeling and Regulation of Metabolic Pathways. Growth on Complementary Nutrients, Biotechnol. Prog., 10, 574-587.
42. Tohyama M., S. Takagi, K. Shimizu (2000). Effect of Controlling Lactate Concentration and Periodic Change in DO Concentration on Fermentation Characteristics of a Mixed Culture of *Lactobacillus delbrueckii* and *Ralstonia eutropha* for PHB Production, J. Biosci. Bioeng., 89, 323-328.
43. Venkatesh K. V., P. Doshi, R. Regaswamy (1997). An Optimal Strategy to Model Microbial Growth in a Multiple Substrate Environment, Biotechnol. Bioeng., 56, 635-644.
44. Wang F., S. Y. Lee (1997). Poly(3-hydroxybutyrate) Production with High Productivity and High Polymer Content by a Fed-batch Culture of *Alcaligenes eutrophus* under Nitrogen Limitation, Appl. Environ. Microbiol., 63, 3703-3706.
45. Wang J., J. Yu (2001). Kinetic Analysis on Formation of Poly(3-hydroxybutyrate) from Acetic Acid by *Ralstonia eutropha* under Chemically Defined Conditions, J. Ind. Microbiol. Biotechnol., 26, 121-126.
46. Yoo S., W.-K. Kim (1994). Cybernetic Model for Synthesis of Poly-β-hydroxybutyric Acid in *Alcaligenes eutrophus*, Biotechnol. Bioeng., 43, 1043-1051.