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ANALYSIS OF CRITICAL MIXING REGIONS TO PROMOTE BIOPOLYMER SYNTHESIS IN FED-BATCH CULTURES OF *WAUTERSIA EUTROPHA*

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ABSTRACT

Despite its advantages over synthetic polymers from petroleum-based feed-stocks, microbially produced poly-β-hydroxybutyrate (PHB) is not commercially competitive owing to low productivities. Productivity enhancement has been explored here through *in silico* experiments with *Wautersia eutropha* in fed-batch fermentations utilizing fructose as the carbon substrate and urea to provide nitrogen. The simulations were based on an experimentally validated kinetic model inserted into mass conservation equations for a bioreactor model that mimicked mixing through an interacting array of three well-mixed tanks. Performance data showed that cell growth and PHB formation were weakly dependent on local mixing in the region of the feed inlets and were determined largely by macromixing in the bulk fluid. However, the best performance depended on an optimum combination of mixing in the three regions, suggest that distributed mixing is an important factor. These results have a physical basis in the metabolism of PHB formation.

KEYWORDS

Poly-β-hydroxybutyrate, *Wautersia eutropha*, Bioreactor, Fed-batch culture, Mixing model, Optimization.

1. INTRODUCTION

In their recent studies, Patwardhan and Srivastava[1,2] and Khanna and Srivastava[3-5] reported experimental and optimization results through different feeding methods to grow *Wautersia eutropha* cultures in fed-batch operation and induce them to synthesize poly-β-hydroxybutyrate (PHB). Their work was motivated by the rapidly growing interest in polyhydroxyalkanoates (PHAs) in general, and PHB in particular, as an alternative to widely used synthetic polymers such as polyethylene (PE) and polypropylene (PP).

The resurgence of interest in PHAs is evident from the many recent reviews[6-8] that have analyzed different aspects of PHA production and purification. As the most prominent member of the PHA family, PHB is an attractive alternative to PE and PP for a number of reasons. Unlike PE and PP, which are synthesized chemically from petroleum-derived feed stocks, PHB can be generated microbially from cheap and/or natural substrates. Microbial synthesis occurs under conditions that are milder than required for the production of PE and PP, and it also consumes less energy[9,10]. In addition to these advantages, many of the properties of PHB, either as a pure polymer or as a co-polymer with polyhydroxyvalerate, are similar to those of chemical polymers[11]. Thus, PHB can replace PE, PP and similar polymers in many applications. PHB scores over these polymers in two other ways: one is its easy biodegradability and the other is its compatibility with body tissues. These benefits extend the potential uses of PHB to environmental, food and medical applications, where the recalcitrance and biological incompatibility of petroleum-based polymers restricts their use[12,13].

Despite its many advantages, the microbial synthesis of PHB has not yet become commercially competitive with PE and PP. A major reason is the low productivity of PHB processes, which offsets their advantages and makes microbial PHB more expensive that chemically produced PE and PP[9,14]. A prime reason for the low productivities is the complexity of the fermentation, which makes conventional modeling and control strategies inadequate and ineffective[7]. Some of the reasons for the complexity are elucidated in section 2, where the mechanism of PHB biosynthesis is described. Many investigators, including Patwardhan and Srivastava[1] and Khanna and Srivastava[3-5], have tried to increase PHB production in fed-batch bioreactors by optimizing the (time-dependent) feed rates of the two primary substrates, one a carbon source and the other a nitrogen source. While significant improvements were obtained, they were not sufficient to make PHB an economically viable substitute for PE and PP.

The problem lies not in the optimization methods but in their application to small laboratory-scale reactors whereas production processes use large vessels, which differ in their performance from laboratory-scale reactors. One major difference is that small bioreactors are usually well-mixed whereas this is rarely possible in large reactors. The impact of mixing of the performances of chemical and biological reactors are well documented[15,16]. These sources inform that complete mixing is desirable to obtain the best performance. However, since complete mixing is practically difficult to achieve in a large reactors, Patnaik and co-workers addressed the problem by asking whether there can be an optimum level of mixing that maximizes a

specified objective, which is usually cell growth or product formation. Interestingly, this turned out to be true for different fermentations[17-19], suggesting that the method may have general validity. Therefore the present work applies the same rationale to increase PHB production by *W. eutropha* through *in silico* experiments with a fed-batch bioreactor.

2. BIOSYNTHESIS OF PHB

PHB is an energy-storage polymer. Both natural and recombinant microorganisms have been used for PHB production, with *Escherchia coli* harboring PHB genes from a wild-type producer being a common recombinant strain. Although recombinant *E. coli* have yielded high concentrations of PHB, their long-term stability and high oxygen demand have limited their use in large-scale fermentations. So, natural strains continue to be preferred, with *W. eutropha* being the most widely used.

The preference for *W. eutropha* (known earlier as *Alcaligenes eutrophus*, *Ralstonia eutropha* and *Cupriavidus necator*[20]) arises from its well-studied physiology, its ease of cultivation, the absence of endo-toxins and the ability of the cells to accumulate large amounts of PHB without impeding their growth[21]. *W. eutropha* synthesizes PHB when subjected to an environment stressful to its growth. In a bioreactor this stress is created by depriving the organism of an essential nutrient such as nitrogen or phosphorus or sulfur. Nitrogen is the most preferred trigger for PHB synthesis because (a) more of nitrogen than sulfur and phosphorus is utilized by the cells, thus providing a strong handle on PHB synthesis and (b) nitrogen deprivation induces the cells to produce more of PHB than do corresponding shortages of the other two elements[8,21]. Although a deficiency of nitrogen is required for PHB synthesis, excessive shortage is harmful since this causes degradation of the biopolymer[22,23].

While the absence of adequate nitrogen may result in high intra-cellular accumulation of PHB, good growth of the cells is required. Good growth obviously requires sufficient amount of carbon. However, excess of carbon is detrimental to growth[3]. Therefore, accurate control of the feed rates of the carbon and nitrogen substrates is the key to the success of the fermentation.

Analyses of the metabolic reactions leading to PHB formation[21,24] have shown that there are three main steps : (i) reversible condensation of AcCoA catalyzed by thiolase, (ii) reduction of AcAcCoA to 3HBCoA by the NADPH-dependent reductase and (iii) assemblage of PHB from 3HBCoA monomer units through the catalytic activity of PHB synthase. All three steps occur at comparable rates, implying that no single step is rate-controlling, all follow complex mechanisms, and there are significant fluxes in both forward and reverse directions. These features complicate optimal control of the flow rates of carbon and nitrogen. The complexities are highlighted by previous optimizations of laboratory-scale bioreactors[4,25,26], which have shown that the absolute as well as relative flow rates of both substrates vary nonlinearly with time and depend on the strain being used.

These difficulties get exacerbated in an imperfectly mixed bioreactor because spatial gradients affect the accessibility of substrates to the cells. As a result, cells in certain regions of the reactor may be swamped by abundant carbon and nitrogen, whereas in other regions the cells may have insufficient availability of either or both substrates. These variations obviously affect the ensemble averages of both biomass and products for a population of cells. Since high productivity of PHB in large bioreactors is of industrial interest, this communication analyzes the effect of mixing on the performance of a population of *R. eutropha* in a fed-batch bioreactor.

3. KINETICS IN AN IMPERFECTLY MIXED BIOREACTOR

Srivastava and associates[1-5] have established a kinetic model for PHB production by *W. eutropha* in a laboratory-scale fed-batch bioreactor. The present analysis is based on their most recent studies[2,5] using *W. eutropha* NRRL B14690. Fructose was the carbon source and urea was the nitrogen source. Fed-batch fermentations were carried out in a 7 L Bioengineering (Switzerland) bioreactor with a starting batch volume of 4 L. Other details are available in their publications[2,5].

In their kinetic model, the total biomass (X) was structured into two components, active biomass (R) and PHB (P), as proposed originally by Mulchandani et al.[27]. The specific growth rate (μ^*) of the biomass was expressed as the product of sigmoidal dependences on the concentrations of the two limiting nutrients, fructose (S_1) and urea (S_2)[28].

$$\mu^* = \mu_m \left[\frac{S_1^{n1}}{S_1^{n1} + K_{s1}^{n1}}\right] \left[\frac{S_2^{n2}}{S_2^{n2} + K_{s2}^{n2}}\right] \quad (1)$$

This equation does not include Khanna and Srivastava's[3] observation that fructose and urea also inhibit cell growth if their concentrations exceed certain threshold values. Including this observation led to a modification of Eq. (1):

$$\mu = \frac{1}{R}\frac{dR}{dt} = \mu^* \left[1 - \left(\frac{S_1}{S_{m1}}\right)^{n3}\right] \left[1 - \left(\frac{S_2}{S_{m2}}\right)^{n4}\right] \quad (2)$$

Since the product (PHB) was formed during both growth and stationary phases, this rate was expressed as :

$$q_p = \frac{1}{R}\frac{dP}{dt} = K_1\mu + K_2 \quad (3)$$

Fructose is utilized by *W. eutropha* for three purposes: growth, polymer synthesis and maintenance. The overall rate of utilization may therefore be represented as :

$$\frac{dS_1}{dt} = \frac{-1}{Y_{R/S1}}\left(\frac{dR}{dt}\right) - \frac{1}{Y_{P/S_1}}\left(\frac{dP}{dt}\right) - m_{s1}R \quad (4)$$

Replacing dP/dt by Eq. (4) leads to:

$$-\frac{dS_1}{dt} = \left(\frac{1}{Y_{R/S1}} + \frac{K_1}{Y_{P/S1}}\right)\frac{dR}{dt} + \left(\frac{K_2}{Y_{P/S1}} + m_{s1}\right)R \quad (5)$$

By rearranging Eq. (5), we obtain the specific rate of fructose consumption (q_{s1}) as:

$$q_{S1} = \frac{1}{R}\frac{dS_1}{dt} = -(\alpha\mu + \gamma) \quad (6)$$

where $\alpha = \frac{1}{Y_{R/S1}} + \frac{K_1}{Y_{P/S1}}$ and $\gamma = \frac{K_2}{Y_{P/S1}} + m_{s1}$

Urea is consumed for the growth of active biomass (R) and for the maintenance functions (m_{S2}) of the cell. Its specific uptake rate may therefore be written as:

$$q_{S2} = \frac{1}{R}\frac{dS_2}{dt} = -\left[\frac{\mu}{Y_{R/S2}} + m_{S2}\right] \quad (7)$$

The kinetic equations may be inserted into the mass balance equations for a fed-batch bioreactor to obtain the overall model for an ideal fermentation[5]:

$$\frac{dR}{dt} = \mu R - \frac{F}{V}R \quad (8)$$

$$\frac{dS_1}{dt} = -(\alpha\mu + \gamma)R + \frac{F_1 S_{01}}{V} - \frac{FS_1}{V} \quad (9)$$

$$\frac{dS_2}{dt} = -\left[\frac{\mu R}{Y_{R/S_2}} + m_{S2}R\right] + \frac{F_2 S_{02}}{V} - \frac{FS_2}{V} \quad (10)$$

$$\frac{dP}{dt} = (K_1\mu R + K_2 R) - \frac{F}{V}P \quad (11)$$

$$\frac{dV}{dt} = F \quad (12)$$

Equations (8)–(12) apply to a perfectly mixed bioreactor. Large bioreactors, where increase in PHB production is really beneficial, are often not perfectly mixed. Many models have been proposed to describe and quantify imperfect (or incomplete) mixing. They are broadly of two types: (a) arrays of well-mixed tanks and (b) dispersion models. The present analysis has used a model of the first kind, proposed by Tosun[29]. His model was based on the segregated-feed-zone model of Villermaux[30]. Although Tosun applied the model to a chemical polymerization process, it is suitable for the PHB production system because, as in Tosun's example, there are two feed streams, each with its own mixing region, and the product is a biopolymer.

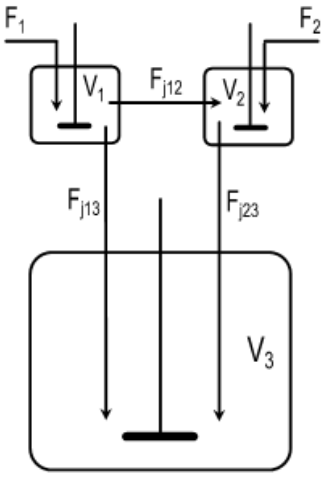


Figure 1

The three-region model of mixing in a fed-batch bioreactor. Reproduced from Tosun[29] with permission from American Institute of Chemical Engineers © 1992.

Figure 1 depicts schematically the Villermaux model[30] as adapted by Tosun[29]. In our application, F_1 represents the feed rate of fructose and F_2 that of urea, with mixing regions V_1 and V_2 respectively. The bulk of the fluid has a volume V_3. The internal flow streams F_{j12}, F_{j13} and F_{j23} represent internal mixing, with the subscript j designating the j-th chemical species. Thus F_{112} is the flow rate of fructose from V_1 to V_2 and F_{212} that of urea from V_1 to V_2. Since V_1 has no urea, $F_{212} = 0$.

As described by Tosun[29], conservation equations may be written for the volumes and the concentrations.

$$\frac{dV_1}{dt} = F_1 - \frac{V_1}{t_M} \quad (13)$$

$$\frac{dV_2}{dt} = F_2 - \frac{V_2}{t_M} \quad (14)$$

$$\frac{dV}{dt} = F_1 + F_2 \quad (15)$$

$$V = V_1 + V_2 + V_3 \quad (16)$$

$$\frac{d(V_1 S_{j1})}{dt} = r_{j1} V_1 + F_1 S_{j1}^0 - F_{j13} - F_{j12} \quad (17)$$

$$\frac{d(V_2 S_{j2})}{dt} = r_{j2} V_2 + F_2 S_{j2}^0 - F_{j23} + F_{j12} \quad (18)$$

$$\frac{d(V_3 S_{j3})}{dt} = r_{j3} V_3 + F_{j13} + F_{j23} \quad (19)$$

The inter-region flow rates may be derived[29,30] to be:

$$F_{j13} = \frac{V_1 S_{j1}}{t_M} + \frac{V_1 (S_{j1} - S_{j3})}{t_1} \quad (20)$$

$$F_{j23} = \frac{V_2 S_{j2}}{t_M} + \frac{V_2 (S_{j2} - S_{j3})}{t_2} \quad (21)$$

$$F_{j12} = \frac{(V_1 + V_2)(S_{j1} - S_{j2})}{t_{12}} \quad (22)$$

Note the presence of four time constants, t_1, t_2, t_{12} and t_m, which are central to the present analysis. The smaller is a time constant, the better is the mixing in that zone. Therefore in the limiting case of all time constants tending to zero, the model reduces to that of perfect mixing, as in the bioreactors used by Srivastava and coworkers[1-5].

Villermaux[30] suggested the following transformations to nondimensionalize the three-region mixing model.

$$\hat{V} = V / V_o; \hat{V}_j = V_j / V_o; \theta_j = t_j / t_0; \hat{F} = t_0 F / V_0;$$

$$\hat{S}_{jk} = S_{jk} / S^0_{jk}, \ \hat{r}_j = r_j t_0 / S_0; \theta = t / t_o$$

Then Eqs. (13)–(22) may be written in dimensionless form as:

$$\frac{d\hat{V}_1}{d\theta} = \hat{F}_1 - \frac{\hat{V}_1}{\theta_M} \quad (23)$$

$$\frac{d\hat{V}_2}{d\theta} = \hat{F}_2 - \frac{\hat{V}_2}{\theta_M} \quad (24)$$

$$\frac{d\hat{V}}{dt} = \hat{F}_1 + \hat{F}_2 \quad (25)$$

$$\hat{V} = \hat{V}_1 + \hat{V}_2 + \hat{V}_3 \quad (26)$$

$$\frac{d(\hat{V}_1 \hat{S}_{j1})}{d\theta} = \hat{r}_{j1} \hat{V}_1 + \hat{F}_1 \hat{S}^o_{j1} - \hat{F}_{j13} - \hat{F}_{j12} \quad (27)$$

$$\frac{d(\hat{V}_2 \hat{S}_{j2})}{d\theta} = \hat{r}_{j2} \hat{V}_2 + \hat{F}_2 \hat{S}^o_{j2} - \hat{F}_{j23} + \hat{F}_{j12} \quad (28)$$

$$\frac{d(\hat{V}_3 \hat{S}_{j3})}{dQ} = \hat{r}_{j3} \hat{V}_3 + \hat{F}_{j13} + \hat{F}_{j23} \quad (29)$$

$$\hat{F}_{j13} = \frac{\hat{V}_1 \hat{S}_{j1}}{\theta_M} + \frac{\hat{V}_1 (\hat{S}_{j1} - \hat{S}_{j3})}{\theta_1} \quad (30)$$

$$\hat{F}_{j23} = \frac{\hat{V}_2 \hat{S}_{j2}}{\theta_M} + \frac{\hat{V}_2 (\hat{S}_{j2} - \hat{S}_{j3})}{\theta_2} \quad (31)$$

$$\hat{F}_{j12} = \frac{(\hat{V}_1 + \hat{V}_2)(\hat{S}_{j1} - \hat{S}_{j2})}{\theta_{12}} \quad (32)$$

As applied to the PHB production system, j =1 represents fructose, j = 2 denotes urea, and j = 3 and 4 represent active biomass and PHB respectively.

Equations (23)–(32) were solved by a fourth-order Runge-Kutta-Gill method using the Berkeley Madonna[31] software and the parameter values listed in Table 1. The intrinsic reaction rates, r_{j1}, r_{j2} and r_{j3}, were represented by the kinetic model of Khanna and Srivastava[5], i.e. Eqs. (2), (3), (6) and (17). Note that since j = 1, 2, 3 and 4, these equations are 10x4=40 in total.

Table 1
Values of the parameters[3,5]

Parameter	Units	Value
α	g g^{-1}	0.48
γ	g g^{-1} h^{-1}	0.0348
μ_m	h^{-1}	0.302
K_1	g g^{-1}	0.008
K_2	g g^{-1} h^{-1}	0.034
K_{S1}	g L^{-1}	22.836
K_{S2}	g L^{-1}	0.234
m_{S1}	g g^{-1} h^{-1}	$5.0*10^{-6}$
m_{S2}	g g^{-1} h^{-1}	$4.5*10^{-6}$
n_1	dimensionless	3.594
n_2	dimensionless	2.213
n_3	dimensionless	3.19
n_4	dimensionless	0.97
S_{01}	g L^{-1}	360
S_{02}	g L^{-1}	12
S_{m1}	g L^{-1}	90.11
S_{m2}	g L^{-1}	10.11
$Y_{P/S1}$	g g^{-1}	1.745
$Y_{R/S1}$	g g^{-1}	16.7
$Y_{R/S2}$	g g^{-1}	1.764

4. RESULTS AND DISCUSSION

Khanna and Srivastava[5] presented results for five combinations of the rates of feeding of fructose and urea. A summary of their results (Table 2) shows that, in terms of the performance of the fermentation, the five cases may be divided broadly into two groups, in each of which the differences between the members are within experimental variations. Therefore one case from the group that yielded higher concentrations of biomass and PHB was chosen for the present analysis. Here Khanna and Srivastava[5] obtained peak concentrations of 35.7 g biomass/L and 18.6 g PHB/L. To understand the effects of fluid mixing on bioreactor performance, each of the time constants θ_1, θ_2 and θ_M was assigned either a low value of 0.01 or a high value of 1.0, thereby generating a set of eight combinations of θ_1, θ_2 and θ_M. As suggested by Tosun[29], the inter-region time constant θ_{12} was set to infinity; implicit in this choice is the assumption that the two feed streams are widely separated, which is reasonable for a large bioreactor. Figures 2 and 3 depict the variations with (dimensionless) time for the volumes of the three mixing regions V_1, V_2 and V_3, as well as the total volume V. In both sets of figures, the plots may be separated into two pairs with distinctly different trends. The two sets of plots on the left side (labeled (a) and (c)), which pertain to a low value of θ_M, decrease with time for V_1 and V_2, and increase for V_3. On the contrary, the plots for a high value of θ_M (labeled (b) and (d)) are almost constant for all three volumes. It is of interest also to note that this dependence on θ_M is independent of the values of θ_1 and θ_2.

The dominant influence of θ_M on the mixing zones may be attributed to at least two factors. One is the high solubilities and diffusivities of the two substrates, fructose and urea, in aqueous media[32,33], as a consequence of which solutions of both substrates quickly get dispersed beyond their zones of injection. The second factor is the increasing significance of macromixing in the bulk liquid as the volume of the reactor increases[34-36]. Therefore θ_1 and θ_2 have little effect on the mixing process, which depends mainly on θ_M. There is also internal consistency between the rates of change of V_1 and V_2. Figures 2 and 3 are compatible with this expectation. The plots for V_1 (fructose) are always above those for V_2 (urea) and, for θ_1 = 0.1, V_2 decays faster than V_1. It has been reported[32,33] that the diffusivity of urea in aqueous media is two orders of magnitude larger than that of fructose. Hence urea may be expected disperse in the bulk of the fermentation broth faster than fructose. The shrinking of V_1 and V_2 is matched by the increase of V_3, thus maintaining conservation of mass within the reactor model.

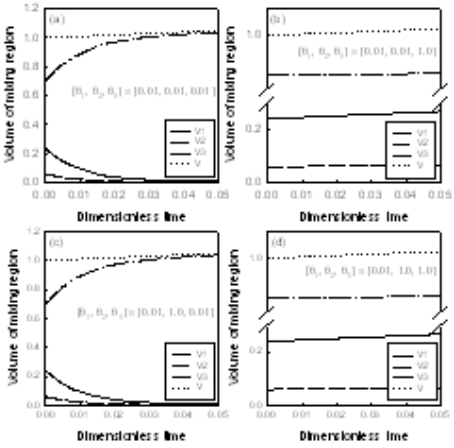


Fig. 2

Time-dependent variations of the volumes of the mixing regions and the total volume of the broth for a low value of θ_1=0.01.

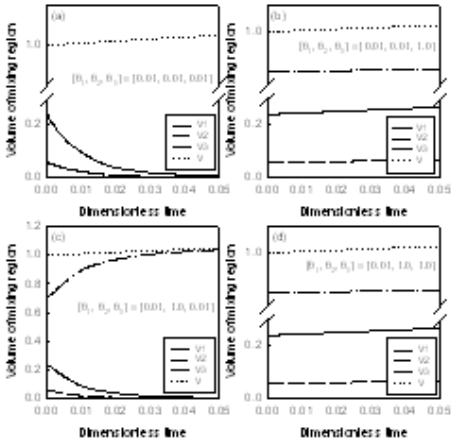


Fig. 3

Time-dependent variations of the volumes of the mixing regions and the total volume of the broth for a high value of θ_1=1.0

Table 2
Results of different simulated feed strategies in fed-batch cultivations[5].

Feed strategy*	Final reactor volume, L	Duration of fermentation, h	Total biomass, g/L	PHB, g/L	PHB, g/L/h	PHB, g/g biomass
Constant S_1 and S_2	5.5	60	35.7	18.6	0.31	0.521
Constant S_1 and S_2	5.2	60	39.5	18.8	0.31	0.476
Constant S_1 and S_2	5.5	60	43.2	22.3	0.37	0.516
Decreasing S_1 and constant S_2	5.2	60	34.4	17.7	0.29	0.514
Decreasing S_1 and S_2	5.12	35	43.0	16.6	0.45	0.386

**Details of the feed rates and concentrations of S_1 (fructose) and S_2 (urea) are given in Khanna and Srivastava[5].*

Mixing in the bioreactor is represented in the model by the flow streams connecting V_1, V_2 and V_3 (Fig.1). The changes in these volumes are the result of the feed streams as well as the inter-region flow rates. These flow rates are plotted in Figs. 4 and 5, and they have one significant similarity and one major difference. Unlike the variations in the volumes of the mixing regions, the qualitative nature of the plots does not change with θ_1 (they are of course independent of θ_1 and θ_2 also). There are, however, significant differences between the flow rates from V_1 to V_2, V_1 to V_3, and V_2 to V_3. The flow rates from V_1 to either V_2 or V_3 decrease monotonically with time but the former drops dramatically within a short span of time, whereas the flow rate from V_1 to V_3 decreases more slowly. This difference may be attributed to the much larger inflow of fructose (about 6-8 times[4,5,25]) than of urea and the greater diffusivity of fructose in aqueous media[32,33]. Because of its high diffusivity, fructose permeates quickly from its own mixing region V_1 to the other two. Now, region V_2 is smaller and rich in urea. Hence, initially the substantial concentration gradient between V_1 and V_2 favors fast transport of fructose towards V_2. However, since V_2 is much smaller than V_1, the former quickly gets saturated with glucose, a process aided by the presence of a high urea concentration. This results in a rapid fall in fructose flow from V_1 to V_2. On the contrary, V_3 is substantially larger than V_1 and it is initially lean in both fructose and urea. Therefore the concentration difference for fructose between V_1 and V_3 is maintained at significant levels over a longer duration of time, resulting in a gradual approach to saturation and correspondingly smooth decreases in the flow rates.

Fig. 4

Variations of the flow rates between the mixing regions for a low value of θ_1=0.01.

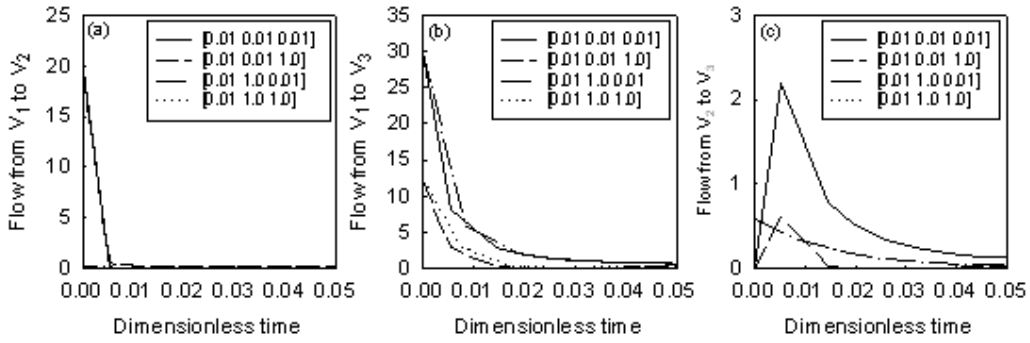
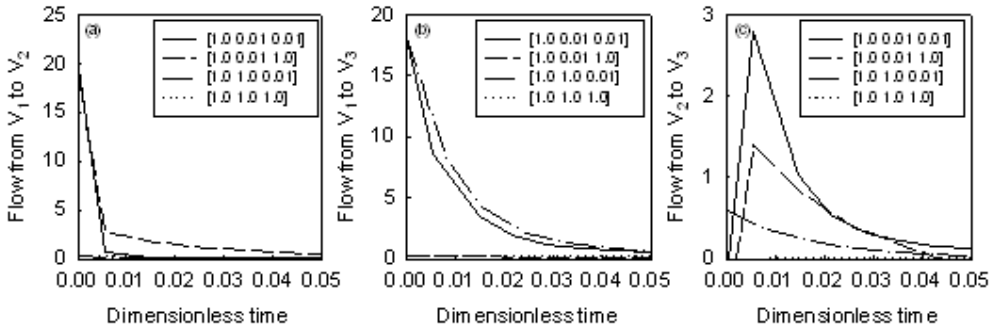


Fig. 5

Variations of the flow rates between the mixing regions for a high value of θ_1=1.0.



The most salient and informative variations are seen in the flow rate from V_2 to V_3. The four plots of this flow rate, in each of Figs. 4 and 5, may be separated into two groups. For θ_2= 0.01, the two plots (one for θ_M= 0.01 and one for θ_M = 1.0) pass through maxima, whereas for θ_2 =1.0 they decrease monotonically and eventually vanish. In fact, for θ_2 = 1.0 and θ_M= 1.0, the flow rates for low as well as high values of θ_1 are so small that they cannot be distinguished from the time-axis. The monotonically decreasing plots are consistent with the trends of the other two internal flow streams. However, when the time constant θ_2 for the urea region (V_2) is small, there is rapid flow from V_2 to V_3, and this is promoted by the initially high flows from V_1 to V_2 (as seen in the left panels in Figs. 4 and 5). As a result, the flow rate from V_2 to V_3 initially increases with time. Soon, however, the flow rate from V_1 to V_2 becomes so small that the initial phase of transfer from V_2 to V_3 cannot be sustained, and consequently the flow rates decrease. This explanation is supported by the observation that the time of attainment of the peaks in the V_2-to-V_3 flow rates is approximately the same as the time at which the V_1-to-V_2 flow rates become negligibly small.

Fig. 6
Variations in the concentration of fructose with time for different combinations of the time constants of the mixing regions.

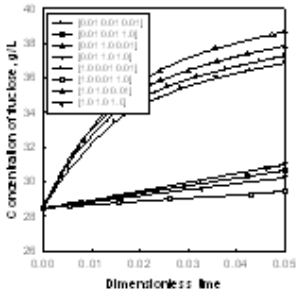
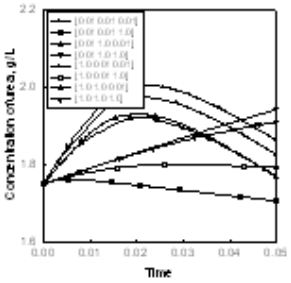


Fig. 7
Variations in the concentration of urea with time for different combinations of the time constants of the mixing regions.



On the basis of this hydrodynamics, Figs. 6-9 portray the variations in four main concentrations that characterize the fermentation. As for the volumes of the mixing regions (Figs. 2 and 3) and the flow rates between them (Figs. 4 and 5), the plots of the concentrations are also seen to be separable into two groups, θ_M = 0.01 and θ_M = 1.0, suggesting mutual consistency between the hydrodynamics and its kinetic manifestation. However, there are also important differences between the profiles of the concentrations. While those for fructose either increase monotonically with time (θ_M = 0.01) or remain nearly constant (θ_M = 1.0), the concentration of urea has very different variations. If θ_M is small, indicating poor mixing, the plots attain maxima at about two-fifth of the duration from the start of the fermentation and then decrease. Intense mixing generates linear variations, whose slopes depend on the value of θ_2. A low time constant θ_2 for the urea- rich region V_2 results in negative slopes whereas a high value of θ_2 (=1.0) generates increasing plots. A similar demarcation is also seen in the profiles of the concentration of active biomass (Fig. 8). This feature has been reported for other microbial system also[17,19,37], indicating that in general any population of cells has an optimum level of mixing that is most favorable to growth.

The variations in PHB concentration (Fig. 9) have distinctive features that may be interpreted in conjunction with the variations of the other concentrations and utilized to design favorable mixing strategies. Unlike the other three variables (Figs. 6-8), the PHB plots for θ_M = 1.0 are coincident with the time-axis,

signifying that almost no PHB is produced for any combination of θ_1 and θ_2. More interestingly, the four plots for θ_M = 0.01 are clearly separable into two types. For θ_2 = 0.01, the concentration of PHB increases with time and eventually stabilizes. If, however, θ_2 is large, there is very little production of PHB. In other words, perfect mixing (as in small bioreactors) is not desirable for efficient production of PHB. A useful inference from this apparently unusual feature is that the incomplete mixing inherent in large (pilot- and industrial-scale) bioreactors may be controlled and exploited for high productivity.

The poor synthesis of PHB when θ_M = 1.0 is biochemically consistent with the variations in the concentration of the nitrogen substrate (urea). It is known[6,7,21] that (a) a shortage of nitrogen induces PHB synthesis by *W. eutropha* and (b) there is some constitutive synthesis in addition to induced synthesis. With good macromixing (θ_M = 1.0), there is no shortage of nitrogen since the concentration of urea increases with time (Fig. 7); hence only constitutive synthesis is possible, and this is normally much smaller than induced synthesis[3,5,26]. With poorer mixing, the urea concentration rises initially but eventually falls as a result of transfer of fluid from V_2 to V_3 (Fig. 7) and the increased consumption due to the growth of biomass (Fig. 8). This fall in urea availability favors induced synthesis of PHB. Thus in the two increasing plots in Fig. 9 (for θ_2 = θ_M = 0.01) the initial rise occurs due to both constitutive and induced synthesis, and the later phase is mainly the result of nitrogen-deprived induction.

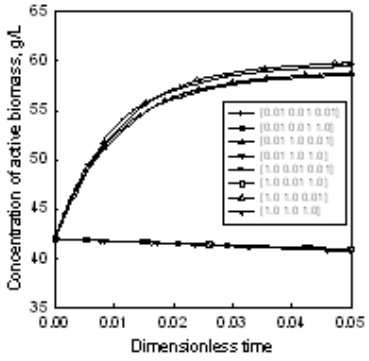


Fig. 8

Variations in the concentration of active biomass with time for different combinations of the time constants of the mixing regions.

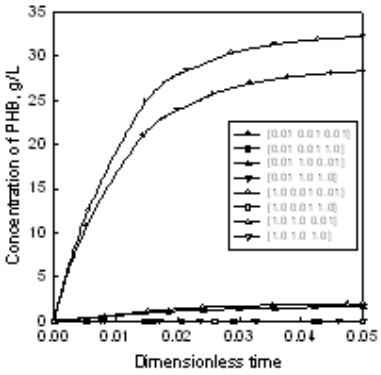


Fig. 9

Variations in the concentration of PHB with time for different combinations of the time constants of the mixing regions.

Two important messages emerge from Figs. 2-9. One is that macromixing in the bulk of the fluid (i.e. θ_M) has a much stronger effect on the fermentation than mixing in the vicinity of entry of the carbon and nitrogen substrates, even though these substrates control the metabolism and kinetics[6-8,21,24]. The second message is that neither complete mixing nor complete segregation desirable, and partial mixing is beneficial.

Although controlled mixing has been shown to enhance the performance of other fermentations also[17,19,37], the reasons may differ from those for PHB synthesis. In the present system, we recognize first that in a well-mixed bioreactor the substrates, products and intermediates are homogeneously distributed. Although the formation of PHB is favored by sufficient amounts of carbon and a shortage of nitrogen, a large excess of carbon and a severe shortage of nitrogen are unfavorable because the former inhibits cell growth[3] while the latter induces depolymerization of PHB[22,23] in addition to promoting its formation. In fact, PHB formation and degradation, both induced by nitrogen-deprivation, from a closed cycle[38], and which of the two processes dominates depends on the extent of shortage of nitrogen.

Maximization of PHB production has a corollary benefit since high intra-cellular concentrations of PHB suppress acetate formation. This is beneficial because acetate, an intermediate formed during PHB biosynthesis, inhibits cell growth. Although maximization of PHB concentration is desirable, this should not be reached too fast since that imposes an unsupportable metabolic load on the cells[39]. Controlling the extent of mixing in the bioreactor helps to balance (a) the synthesis and degradation of PHB and (b) PHB production and acetate formation. The results of the present study through a mixing model support this conclusion and corroborate similar findings obtained though a dispersion model[25,40].

5. CONCLUSIONS

Enhancement of the productivity of poly-β-hydroxybutyrate (PHB) in large-scale fermentations requires optimization of the degree of mixing in the bioreactor. Neither complete homogeneity nor predominantly segregated flow is desirable, and neither is present under normal operating conditions.

Investigation of the simulated performance of a fed-batch bioreactor for PHB production by *W. eutropha* utilizing a nitrogen feed (urea) and a carbon feed (fructose) showed that the output of biopolymer depends on fluid mixing in three regions: two around the inlet ports of the feed streams and the third in the remainder of the bulk fluid. Fluid flow between the regions was depicted by a model from the literature, with each region characterized by a time constant.

Although the evolution of biomass and PHB concentrations with time, and their peak values, depended on all three time constants, their dependence on the local mixing rates for fructose and urea was weaker than for the bulk fluid. This difference agrees with observations from other fermentations that macromixing is the controlling factor in large bioreactors. Significantly, however, maximization of PHB production required optimizing not just bulk mixing but in the other two regions as well. Such an optimization resulted in maximum attainable concentrations of 57.8 g biomass/L and 28.3 g PHB/L, which represent increases of 61.7% and 52.1% over Khanna and Srivastava's [5] results with a well-mixed bioreactor. These results suggest that optimizing a complex fermentation, such as that for PHB, in a large bioreactor requires optimizing mixing in certain critical regions rather in the broth as a whole.

NOMENCLATURE

θ_M = dimensionless mixing time

θ = dimensionless real time

θ_1 = dimensionless time constant for V_1

θ_2 = dimensionless time constant for V_2

θ_{12} = dimensionless value of 12

μ = corrected specific growth rate, h^{-1}

μ^* = uncorrected specific growth rate, h^{-1}

μ_m = maximum specific growth rate, h^{-1}

F_1 = feed rate of glucose, L h^{-1}

F_2 = feed rate of urea, L h^{-1}

F = combined feed rate, L h^{-1}

F_{jkl} = flow rate of S_{jk} from k-th to l-th . mixing region, L h^{-1}

$\hat{F}$ = dimensionless total inflow Ft_0 / V_0

$\hat{F}_j$ = dimensionless inflow of j-th species $F_j t_0 / V_0$

K_1 = growth-associated rate constant for PHB, g g^{-1}

K_2 = non-growth-associated rate constant for PHB, g $g^{-1}h^{-1}$

K_{s1} = equilibrium constant for S_1, g L^{-1}

K_{s2} = equilibrium constant for S_2. g L^{-1}

m_{S1} = maintenance coefficient for fructose, g $g^{-1}h^{-1}$

m_{S2} = maintenance coefficient for urea, g $g^{-1}h^{-1}$

n_1 = exponent for S_1 in Eq.(1)

n_2 = exponent for S_2 in Eq. (1)

n_3 = exponent for S_1 in Eq. (2)

n_4 = exponent for S_2 in Eq. (2)

P = concentration of PHB, g L^{-1}

q_p = specific rate of formation of PHB, h^{-1}

q_{S1} = specific uptake rate of glucose, h^{-1}

q_{S2} = specific uptake rate of urea h^{-1}

Q = dimensionless time constant t / t_0

Q_1 = dimensionless time constant t_1 / t_0

Q_2 = dimensionless time constant t_2 / t_0

Q_{12} = dimensionless time constant t_{12} / t_0

Q_m = dimensionless mixing time t_m / t_0

$\hat{r}_{jk}$ = dimensionless rate $r_{jk} t_0 / S^0_{jk}$

r_{jk} = rate of change of S_{jk}, g L^{-1} h^{-1}

R = concentration of active biomass, g L^{-1}

S_1 = concentration of fructose, g L^{-1}

S_2 = concentration of urea, g L^{-1}

S_{m1} = limiting value of S_1, g L^{-1}

S_{m2} = limiting value of S_2, g L^{-1}

$\hat{S}_{jk}$ = dimensionless concentration S_{jk} / S^0_{jk}

S_{jk} = concentration of j-th species in k-th mixing region, g L^{-1}

S^o_{jk} = initial concentration of j-th species in k-th mixing region, g L^{-1}

t = elapsed time, h

t_0 = time of starting fed-batch operation, h

t_M = mixing time in the bioreactor, h

t_1 = time constant for V_1, h

t_2 = time constant for V_2, h

t_{12} = time constant for transfer from region- to region-2, h

V = volume of material in the bioreactor, L

V_1 = volume of fructose mixing

region, L

V_2 = volume of urea mixing region, L

V_3 = remaining volume of the broth, L

$\hat{V}$ = dimensionless volume V / V_0

$\hat{V}_j$ = dimensionless j-th region volume V_j / V_0

V_0 = volume of the broth at t = t_0, L

$Y_{P/S1}$ = yield coefficient for PHB with respect to fructose, g g^{-1}

$Y_{R/S2}$ = yield coefficient for active biomass with respect to urea, g g^{-1}

$Y_{R/S1}$ = yield coefficient for active biomass with respect to fructose, g g^{-1}

REFERENCES

1. Patwardhan PR and Srivastava AK, Model based fed-batch cultivation of *R. eutropha* for enhanced biopolymer production. Biochem Eng J 20: 21-28 (2004).
2. Patwardhan PR and Srivastava AK, Fed-batch cultivation of *Wautersia eutropha*. Bioresour Technol 99: 1787-1792 (2008).
3. Khanna S and Srivastava AK, A simple structured model for biopolymer (PHB) production. Biotechnol Prog 21: 830-838 (2005).
4. Khanna S and Srivastava AK, Computer-simulated fed-batch cultivation for over production of PHB: A comparison of simultaneous and alternate feeding of carbon and nitrogen. Biochem Eng J 27: 197-203 (2006).
5. Khanna S and Srivastava AK, Productivity enhancement of poly-(β-hydroxybutyrate) by fed-batch cultivation of nutrients using variable (decreasing) nutrient rate by *Wautersia eutropha.* Chem Eng Commun 195: 1424-1436 (2008).
6. Khanna S and Srivastava AK, Recent advances in microbial polyhydroxyalkanoate. Process Biochem 40: 607-619 (2005).
7. Patnaik PR, Perspectives in the modeling and optimization of PHB production by pure and mixed cultures. Crit Revs Biotechnol 25: 153-171 (2005).
8. Dias JML, Lemos PC, Serafim LS, Oliviera C, Eiroa M, Alberquerque MGE, Ramos AM, Oliviera R and Reis MAM, Recent advances in polyhydroxyalkanoate production by mixed aerobic cultures: From the substrate to the final product. Macromol Sci 6: 885-906 (2006).
9. Nonato RV, Mantellato PE and Rossell CEV, Integrated production of biodegradable plastic, sugar and ethanol. Appl Microbiol Biotechnol 57: 1-5 (2001).
10. Harding KG, Dennis JS, von Blottinz H and Harrison STL, Environmental analysis of plastic production process: comparing petroleum-based polypropylene and polyethylene with biologically-based poly-beta-hydroxybutyric acid using life cycle analysis. J Biotechnol 130: 57-66 (2007).
11. van der Walle GAM, de Koning GJM, Weusthius RA and Eggink G, Properties, modifications and applications of bio-polyesters. Adv Biochem Eng Biotechnol 71: 262-291 (2001).
12. Bucci DZ, Tavares LBB and Sell I, PHB packaging for the storage of food products. Polym Test 24: 564-571 (2005).
13. Doi Y, Microbial Polyesters. VCH, New York (1990).
14. Choi JI and Lee SY, Process analysis and economic evaluation for poly (3-hydroxybutyrate) production by fermentation. Bioprocess Eng 17: 335-342 (1997).

15. Hadjiev D, Sabiri NE and Zanati A, Mixing time in bioreactors under aerated conditions. Biochem Eng J 27: 323-330 (2006).
16. Rao DG, Introduction to Biochemical Engineering. Tata-McGraw Hill, New Delhi (2005).
17. Patnaik PR, On the improvement of bacterial growth on complementary substrates by partial segregation in the broth. J Chem Technol Biotechnol 75: 229-236 (2000).
18. Ganduri VSRK, Ghosh S and Patnaik PR, Mixing control as a device to increase PHB production in batch fermentations with co-cultures of *Lactobacillus delbrueckii* and *Ralstonia eutropha.* Process Biochem 40: 257-264 (2005).
19. Patnaik PR, Effect of fluid dispersion on cybernetic control of microbial growth on substitutable substrates. Bioprocess Eng 25: 315-321 (2003).
20. Vandamme P and Coenye T, Taxonomy of the genus Cupriavidus : A tale of lost and found. Int J Syst Evol Microbiol 54: 2285-2289 (2004).
21. Babel W, Ackermann JU and Breuer U, Physiology, regulation and limits of the synthesis of poly(3HB). Adv Biochem Eng Biotechnol 71: 125-157 (2001).
22. Handrick R, Reinhardt S and Jendrossek D, Mobilization of poly(3-hydroxybutyrate) in *R. eutropha.* J Bacteriol 182: 5916-5918 (2000).
23. Jendrossek D, Microbial degradation of polymers. Adv Biochem Eng Biotechnol 71: 293-325 (2001).
24. Leaf TA and Srienc F, Metabolic modeling of polyhydroxybutyrate biosynthesis. Biotechnol Bioeng 57: 557-570 (1998).
25. Patnaik PR, Dispersion optimization to enhance PHB production in fed-batch cultures of *Ralstonia eutropha.* Bioresour Technol 97: 1994-2001 (2006).
26. Groethe E, Moo-Young M and Chisti Y, Fermentation optimization for the production of poly(β-hydroxybutyric acid) microbial thermoplastic. Enzyme Microb Technol 25: 132-141 (1997).
27. Mulchandani A, Luong JHT and Groom C, Substrate inhibition Kinetics for microbial growth and synthesis of poly-β-hydroxybutyric acid by *Alcaligenes eutrophus* ATCC 17697. Appl Microbiol Biotechnol 30: 11-17 (1989).
28. Heinzle RM and Lafferty A, A mathematical model for growth and synthesis of poly-β-hydroxybutyric acid (PHB) in *Alcaligenes eutrophus* H16. Eur J Appl Microbiol 11: 8-16 (1980).
29. Tosun G, A mathematical model of mixing and polymerization in a semibatch stirred-tank reactor. A I Ch E J 38: 425-437 (1992).
30. Villermaux J, A simple model for partial segregation in a semi-batch reactor. Paper 114a, AIChE Meeting, San Francisco, CA (1989).
31. Macey R and Oster G, Berkeley Madonna. Modeling and Analysis of Dynamic Systems. http:www.berkeleymadonna.com/ (2001).
32. Araujo MA, Ferreira EC, Cunha AM and Mota M, Determination of diffusion coefficients of glycerol and glucose from starch based thermoplastic compounds on simulated physiological solution. J Matl Sci Matl Med 16: 239-246 (2005).
33. Idrissi A, Cinar E, Longelin S and Damay P, The effect of temperature on urea-urea interactions in water : a molecular dynamics simulation. J Mol Liqs 110: 201-208 (2004).
34. Micheletti M, Barrett T, Doig SD, Baganz F, Levy MS, Woodley JM and Lye GJ, Fluid mixing in shaken bioreactors: Implications for scale-up predictions from microlitre-scale microbial and mammalian cell cultures. Chem Eng Sci 61: 2939-2949 (2006).
35. Oriscu C, Galaction A-I, Cascaval D and Ungureau F, Modeling of mixing in stirred bioreactors. 2. Mixing time for non-

aerated broths. Biochem Eng J 12: 61-69 (2002).
36. Simmons MJH, Zhu H, Biyalski W, Hewitt CJ and Nienow AW, Mixing in a model bioreactor using agitators with a high solidity ratio and deep blades. Chem Eng Res Des 85: 551-559 (2007).
37. Ganduri VSRK, Ghosh S and Patnaik PR, Optimal mixing to improve the performance of batch and continuous fermentations with recombinant *Escherichia coli.* J Chem Technol Biotechnol 80: 361-370 (2005).
38. Doi Y, Segawa A, Kawaguchi Y and Kunioka M, Cyclic nature of poly (3-hydroxyalkanoate) metabolism in *Alcaligenes eutrophus.* FEMS Microbiol Lett 67: 165-170 (1990).
39. Wang J and Yu J, 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 (2001).
40. Patnaik PR, Analysis of the effect of flow interruptions on fed-batch fermentation for PHB production by *Ralstonia eutropha* in finitely dispersed bioreactors. Chem Eng Commun 194: 603-617 (2007).