

Genetic Algorithmic Optimization of PHB Production by a Mixed Culture in an Optimally Dispersed Fed-batch Bioreactor^{*}

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Abstract: Poly- β -hydroxybutyrate (PHB) is an energy-storage polymer whose properties are similar to those of chemical polymers such as polyethylene and polypropylene. Moreover, PHB is biodegradable, absorbed by human tissues and less energy-consuming than synthetic polymers. Although *Ralstonia eutropha* is widely used to synthesize PHB, it is inefficient in utilizing glucose and similar sugars. Therefore a co-culture of *R. eutropha* and *Lactobacillus delbrueckii* is preferred since the latter can convert glucose to lactate, which *R. eutropha* can metabolize easily. Tohyama et al. [24] maximized PHB production in a well-mixed fed-batch bioreactor with glucose and $(\text{NH}_4)_2\text{SO}_4$ as the primary substrates. Since production-scale bioreactors often deviate from ideal laboratory-scale reactors, a large bioreactor was simulated by means of a dispersion model with the kinetics determined by Tohyama et al. [24] and dispersion set at an optimum Peclet number of 20 [32]. The time-dependent feed rates of the two substrates were determined through a genetic algorithm (GA) to maximize PHB production. This bioreactor produced 22.2% more PHB per liter and 12.8% more cell mass than achieved by Tohyama et al. [24]. These results, and similar observations with other fermentations, indicate the feasibility of enhancing the efficiency of large nonideal bioreactors through GA optimizations.

Keywords: Poly- β -hydroxybutyrate; Mixed culture; Nonideal bioreactor; Optimum dispersion; Genetic algorithm.

Introduction

The rapidly growing number of publications in recent years [1-3] indicates a corresponding growth in research interest in the microbial production of polyhydroxyalkanoates (PHAs). After many years of stagnation, this resurgence of interest has been motivated by a number of factors, mainly the fluctuations and uncertainty in the availability and prices of petroleum-derived substrates and in the rapidly increasing levels of environmental pollution [4, 5]. PHAs are homo- or hetero-polyesters that are synthesized by different prokaryotes under stressful conditions. The stresses are usually in the form of shortages of one or more essential ingredients for cell metabolism, which trigger the synthesis of PHAs as energy reservoirs [6-8].

Poly- β -hydroxybutyrate (PHB) is possibly the most significant PHA and thus much of the interest in PHAs is focused on PHB [3]. The petrochemical and environmental concerns mentioned above are relevant to the biosynthesis of PHB because this polymer is a potential replacement for polymers such as polypropylene (PP) and polyethylene (PE) that are currently synthesized chemically from petroleum-derived reactants. PHB can replace these polymers since many of its properties, sometimes as a copolymer with polyhydroxyvalerate, are as good

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as or better than those of PE and PP [9, 10]. Moreover, PHB can be synthesized under milder and less energy-consuming conditions than PE and PP, utilizing renewable resources as against non-renewable petroleum feed stocks for the latter polymers. Unlike chemical polymers, PHB is easily biodegraded and it is compatible with many tissues in the human body, thus widening its uses beyond those of PE, PP and similar polymers.

In theory, therefore, the competition between PHB and polymers derived from petroleum-based reactants should have favored PHB for industrial production. Petroleum-based polymers however continue to dominate the market because the low productivity and high cost of microbial PHB over-ride its beneficial properties [4, 11]. Since energy consumption is low and raw materials are not expensive, the unfavorable cost of microbial PHB arises mainly from the low yields, due to inadequate understanding of the fermentation under production conditions and ineffective modeling and optimization [2, 12].

Owing to the complexity of the metabolism of PHB biosynthesis [6, 8] and the insufficiently understood effects of environmental noise, mathematical models constructed on the basis of 'clean' laboratory-scale experiments often become inadequate and/or inapplicable under real conditions [2, 13]. In such situations, models based on methods of artificial intelligence (also called cognitive models) have been more efficient in describing and optimizing bioreactor behavior. Several so-called 'intelligent' methods are available, and each has both advantages and limitations [14, 15].

Recent studies suggest that genetic algorithms (GAs) are effective in portraying and controlling the performance of microbial reactors under different conditions. They have been particularly useful for fed-batch bioreactors, where time-dependent control of one or more feed streams is critical and the process is inherently unsteady. Owing to their stochastic nature, their multi-solution strategies, their evolutionary approach and their lack of dependence on process models, GAs avoid typical weaknesses of classical optimization such as the singularities of analytic methods [16] and convergence difficulties of numerical methods [17]. Applications to fermentation systems with different microorganisms and different kinetics demonstrate the effectiveness and versatility of GAs in optimizing fed-batch microbial cultures. Na et al. [18] optimized a fed-batch culture for salmon calcitonin, an intracellular protein synthesized by a recombinant *Saccharomyces cerevisiae* strain. Later, Sarkar and Modak [19] illustrated the ability of a GA to circumvent the singularities contained in a fed-batch model by optimizing the feed rates to enhance (a) the production of a secreted recombinant protein and (b) the growth of *S. cerevisiae* cells. Roeva et al. [20] and Roeva and Tzonkov [21] maximized phytase production by *Escherichia coli* containing the plasmid pPhyt109 through feed rate optimization by means of a GA. Lee et al.'s [22] work demonstrates the power of the GA approach for a complex industrial-scale problem. Their system comprised a train of fed-batch bioreactors operated synchronously to produce penicillin V by *Penicillium crysogenum*. They also had to satisfy a number of inter-linked optimization functions simultaneously. To succeed in these applications, the investigators had to recruit additional features into the GAs employed; these included elitism [18], on-line variations in the crossover and mutation steps [19], and a combination of elitism and diversity preservation [22].

However, there is yet no report of the application of a GA method to PHB biosynthesis in spite of the complexity of its metabolism and operating procedure [16, 23], and the need to increase the yield of PHB to make it competitive with chemically synthesized PP and PE. Because of these reasons and the successes of GAs in other bioreactor applications, the

present study has applied a GA method to optimize a fed-batch bioreactor producing PHB through a co-culture of *Ralstonia eutropha* and *Lactobacillus delbrueckii*. The next section describes the advantages of this co-culture and its kinetics, followed by an account of the GA procedure and then a comparison of the results with the analytic optimization results of Tohyama et al. [24].

PHB production by a co-culture

Owing to its well-understood physiology, its ease of fermentation and downstream processing, and its ability to accumulate large concentrations of PHB inside the cells (up to 80-90% of dry cell weight [3, 25], *R. eutropha* is a preferred organism for PHB synthesis, despite competition from other organisms such as *Alcaligenes latus*, *Azetobacter vivelandii* and recombinant *Escherichia coli*.

R. eutropha (formerly known as *Alcaligenes eutrophus* and recently renamed *Cupriavidus necator* and *Wautersia eutropha*) synthesizes PHB under conditions that are adverse to its growth [6-8]. Such conditions arise when the organism is deprived of an essential substrate such as nitrogen or phosphorus. Deprivation of nitrogen is preferred because inexpensive nitrogen sources (such as ammonium salt or urea) are readily available and the lack of nitrogen induces greater amounts of PHB formation than a shortage of phosphorus or sulfur. Although carbon is also an essential substrate, curtailment of carbon supply is not desirable since this reduces cell growth and consequently the volumetric output of PHB in the fermentation.

Kinetic studies of PHB biosynthesis [16, 26, 27] indicate that *R. eutropha* requires two key substrates: a carbon source, which is usually fructose or glucose or lactose, and a nitrogen source such as NH_4Cl or $(\text{NH}_4)_2\text{SO}_4$ or urea. Carbon contributes to the growth of the cells, and hence there should always be an adequate supply of carbon to the cells. Although nitrogen too is associated with growth, the absence of nitrogen initiates PHB synthesis. This happens because PHB is an energy-storage polymer, and the lack of nitrogen induces the cells to synthesize an alternative energy reservoir. Although sufficient carbon and insufficient nitrogen are key factors determining cell growth and PHB formation, excess of carbon suppresses cell growth [26] and a severe shortage of nitrogen is detrimental to both growth and stability of intra-cellular PHB [26, 28]. Under such conditions, fed-batch fermentation is the most effective mode of operation [1, 3].

Although *R. eutropha* synthesizes PHB from glucose and similar substrates, the uptake and metabolism are too slow for a viable process. On the contrary, the organism can utilize organic acids such as acetate, butyrate and lactate much faster. However, since these acids are relatively expensive, it is preferable to generate them *in situ* during a fermentation. This is done by co-culturing *R. eutropha* with another organism that is efficient in utilizing glucose or lactose. Katoh et al. [29] and Tohyama and Shimizu [30] showed that *Lactobacillus delbrueckii* is such an organism, and co-cultures of *L. delbrueckii* with *R. eutropha* were therefore more efficient in utilizing glucose and producing PHB than *R. eutropha* alone. Their results, supplemented by independent observations, were modeled by Tohyama et al. [24], and their model has been used in the present study. Detailed equations of the model are presented in the Appendix. As in their earlier work [30], Tohyama et al. [24] used *L. delbrueckii* IAM1928 to produce lactate as an intermediate that was converted by *R. eutropha* to PHB. Glucose was the carbon source and $(\text{NH}_4)_2\text{SO}_4$ the nitrogen source. Fermentations were done in a 5 l jar fermentor (MDL500-5L, Marubishi, Tokyo, Japan) with a working

volume of 3 l, maintained at 37⁰C. Details of the measurement techniques, assay methods and the fermentation medium are described by the authors.

Owing to the differences between *R. eutropha* and *L. delbrueckii*, the mixed culture model differs from those for *R. eutropha* as a single culture in a number of features. Since *R. eutropha* is aerobic, it is possible to supply oxygen (or air) continuously, allowing the DO content to go even up to saturation [2, 26, 31]. This obviously cannot be allowed for the present co-culture since *L. delbrueckii* is anaerobic. As explained before, Tohyama and Shimizu [30] and Tohyama et al. [24] overcame this problem by initially maintaining a low value (of 0.5 ppm) of the DO to suit *L. delbrueckii* and then increasing the DO level (to 3ppm) after there has been sufficient growth and lactate formation.

Since *R. eutropha* prefers lactate over glucose and fructose, the fermentation is inoculated initially with *L. delbrueckii* in order to convert the sugars to lactate; *R. eutropha* is then introduced after a sufficient amount of lactate has been formed. Tohyama and coworkers inoculated *R. eutropha* about 3 h after the cultivation of *L. delbrueckii* started; at this time the lactate concentration had risen to about 3 g·l⁻¹. Obviously, PHB formation started only after inoculation by *R. eutropha*.

The presence of lactate is another key feature that differentiates this co-culture from a pure culture. Since lactate is a product from *L. delbrueckii* and a substrate for *R. eutropha*, it exercises feedback control on the fermentation system. Substrate inhibition is a key feature of this control in that lactate favors PHB biosynthesis up to a critical concentration but becomes inhibitory after that. According to Tohyama et al.'s model, this concentration is $\sqrt{K_p K_l}$; however, their bioreactor had almost complete dispersion and hence the critical lactate concentration in the presence of finite dispersion may differ.

The presence of finite dispersion was accounted here by means of standard mass balances for each of the concentrations in terms of the Peclet number *Pe*. The degree of dispersion may be increased by changing the value of *Pe*. Tohyama et al.'s [24] results could be replicated by setting *Pe* → 0, signifying complete dispersion. For the present analysis, the optimum value of *Pe* = 20 [32] was used; such a positive finite dispersion is characteristic of large bioreactors.

The optimization problem

Optimization of a fed-batch bioreactor for PHB production by a co-culture involves determination of the time-varying feed rates of the nitrogen and carbon substrates that together maximize PHB concentration at the end of the fermentation. Without dispersion limitation (i.e. with *Pe* → 0), the mass balances are of the form:

$$\frac{d(X_1V)}{dt} = r_1V = \mu_1 X_1V \quad (1)$$

$$\frac{d(X_2V)}{dt} = r_2V = \mu_2 X_2V \quad (2)$$

$$\frac{d(PV)}{dt} = r_pV = (\sigma_1 X_1 - \nu_2 X_2)V \quad (3)$$

$$\frac{d(SV)}{dt} = F_S S_O + r_S V = F_S S_O - \nu_1 X_1 V \quad (4)$$

$$\frac{d(NV)}{dt} = F_N N_O + r_N V = F_N N_O - \nu_3 X_2 V \quad (5)$$

$$\frac{d(QV)}{dt} = r_Q V = \sigma_2 X_2 V \quad (6)$$

$$\frac{dV}{dt} = F_S + F_N \quad (7)$$

The reaction rates r_1, r_2, r_S, r_P, r_N and r_Q follow Eqs. (21)-(24), (31) and (32), specified in the Appendix. The rate of increase of the volume, V , of the contents of the bioreactor is the total inflow rate ($F_S + F_N$).

The aim is to maximize PHB output at the end of a time point t_f (the final time), which may be expressed as:

$$\text{Maximize } I = (QV)_{t_f} \quad (8)$$

subject to the constraints:

$$V(t) \leq V_{\max} \quad (9)$$

$$F_{S,\min} \leq F_S \leq F_{S,\max} \quad (10)$$

$$F_{N,\min} \leq F_N \leq F_{N,\max} \quad (11)$$

$$X_1 > 0, X_2 > 0, S > 0, N \geq 0, P \geq 0, Q > 0 \quad (12)$$

The upper limit on V is set by the volume of the bioreactor (usually $V_{\max} \approx 0.7 \cdot \text{reactor volume}$). Eq. (12) formalizes the physical requirement that all concentrations should be non-negative. In addition, while the culture concentrations and those of glucose and PHB have to be greater than zero for a feasible fermentation, the concentrations of $(\text{NH}_4)_2\text{SO}_4$ and lactate are allowed to vanish for short durations without damaging the process. To ensure that there is no prolonged absence of these two components, the maximum allowable durations for them to vanish may be specified in advance. If these time limits are exceeded, the corresponding concentration (N or P) is set to zero. The physical interpretation of this numerical strategy is that a small dose of either $(\text{NH}_4)_2\text{SO}_4$ or lactate is injected if the concentration remains undetectable for a time interval longer than allowed.

In principle, the optimization problem posed by Eq.(8) may be solved by an analytic method such as Pontryagin's maximum principle even though methods have to be devised [16, 19] to overcome the singularities arising out of the linear presence of F_S and F_N in Eqs.(4), (5) and (7).

Analytic optimization methods, however, become intractable if there is finite dispersion in the fermentation broth. Then the mass balances may be written vectorially as:

$$\frac{1}{D} \frac{\partial \bar{y}}{\partial t} = \frac{1}{Pe} \frac{\partial^2 \bar{y}}{\partial z^2} - \frac{\partial \bar{y}}{\partial z} \pm \frac{1}{D} \bar{r} \quad (13)$$

where \bar{y} contains the concentrations X_1, X_2, S, P, N and Q . Pe is the dimensionless Peclet number, defined as:

$$Pe = \frac{uR}{D_e}$$

For a fully dispersed broth, the effective dispersion coefficient $D_e \rightarrow \infty$ and consequently $Pe \rightarrow 0$; this was the case in Tohyama and Shimizu [30] and Tohyama et al. [24]. If there is no dispersion, as in plug flow, $D_e \rightarrow 0$ and $Pe \rightarrow \infty$. Large bioreactors usually have finite non-zero values of D_e and Pe ; in the present work, Pe was set at the optimum value of 20 [32].

Eq. (13) is subject to the following initial and boundary conditions:

$$t = 0 \quad : \quad \bar{y} = \bar{y}_0 \quad \forall z \quad (\text{homogeneity}) \quad (14)$$

$$z = 0 \quad : \quad \frac{\partial \bar{y}}{\partial z} = 0 \quad (\text{axial symmetry}) \quad (15)$$

$$z = R \quad : \quad \frac{\partial \bar{y}}{\partial z} = 0 \quad (\text{no outflow}) \quad (16)$$

Now the maximization problem, Eq. (8), has to be solved in conjunction with Eqs. (13)-(17) and the kinetic equations spelt out in the Appendix. This is a formidable task either analytically or numerically; alternate approaches such as Monte Carlo methods or those utilizing artificial intelligence (AI) offer more convenient ways to solve the problem. Genetic algorithms are a class of AI methods. Based on many previous applications [18-21, 33], the present investigation has employed a genetic algorithm to the fed-batch PHB optimization problem with dispersion set at $Pe = 20$, determined to be optimum in an earlier study [32].

The application of a GA to a boundary value problem such as that defined by Eqs. (13)-(16) also differs from its application to an initial value problem [Eqs. (1)-(7)] in that for the latter case analytic expressions may be proposed for the singular feed rates of the two primary substrates. Following Sarkar and Modak [19], they may be expressed as:

$$F_S^* = \frac{v_1 X_1 V}{S_O - S} + \Delta F_{SC} X_1 V \quad (17)$$

$$F_N^* = \frac{v_3 X_2 V}{N_O - N} + \Delta F_{NC} X_2 V \quad (18)$$

The first term in each equation represents the feed rate required to maintain a constant substrate concentration, and the second term is a 'correction' that accounts for variation of the concentration in the singular interval. The two first terms in Eqs. (17) and (18) may be determined numerically and then improved by adding the correction terms. The form of the correction terms proposed by Sarkar and Modak [19] is sufficiently general to be usable in the present case, so it may be retained as:

$$\Delta F_{SC} = a_S S^{b_S} P^{c_S} Q^{d_S} \quad (19)$$

$$\Delta F_{NS} = a_N N^{b_N} P^{c_N} Q^{d_N} \quad (20)$$

The values of the parameters a_S , a_N , etc. are calculated and updated during each stage of the GA.

Genetic algorithm methodology

The theory of genetic algorithms (GAs) is well documented [34, 35] and hence only a concise introduction is provided here in the context of the actual application. Unlike analytic and numerical optimization methods, but like simulated annealing and some forms of fuzzy neural networks, GAs are stochastic search techniques that begin with a population of likely solutions and generate step-wise the one solution that satisfies the prescribed optimization criterion best of all. The route from the starting population to the optimum solution uses at each stage what are called fitness functions that are conceptually similar to the membership functions in fuzzy logic.

The present optimization problem requires determination of the time-dependent feed rates of $(\text{NH}_4)_2\text{SO}_4$ and glucose that maximize the concentration of PHB at the end of a specified duration of time. We note here that the final time, t_f , has been fixed in advance. t_f may also be variable, in which case it is determined as part of the optimization problem. In the present study t_f was fixed *a priori* at 30 h in order to match Tohyama et al.'s [24] choice and compare the present results with theirs.

The first step in implementing a GA is to encode the decision variables as strings that are referred to as chromosomes or genomes. Now, two key substrates are fed to the bioreactor: glucose and $(\text{NH}_4)_2\text{SO}_4$. According to the optimum control theory referred to earlier, each feed rate will consist of intervals of maximum, singular and minimum values. Following Sarkar and Modak [19], these may be encoded by the integers 1, 2 and 3 respectively. Then the first three members of each genome in the starting population comprise a set of integers generated randomly from the set [1, 2, 3]. These members are called genes. These genes are followed by the values of the unknown coefficients a_j , b_j , c_j , and d_j in the feed correction terms [Eqs. (19) and (20)] where $j = S$ (for glucose) or N (for $(\text{NH}_4)_2\text{SO}_4$). These genes may be determined in a similar manner by generating real numbers randomly for the coefficients within specified lower and upper limits. The third (and final) part of a genome has the switching times between the intervals; the length, L of this part will therefore depend on the number of intervals, n_I , between the starting time, t_0 , and the final time, t_f . In our case, since t_f has been fixed in advance, $L = n_I - 1$. The total length of each genome is thus $n_T = 2n_I + n_C - 1$, where n_C is the number of unknown coefficients in the feed correction term. Since $n_C = 4$ here, a typical genome may have the following sequence of genes:

$$1213 \mid a_j b_j c_j d_j \mid t_1 t_2 t_3 t_4 t_5$$

The first four numbers are randomly generated integers from the set [1-3], as explained above. The next four genes represent the coefficients, and the last five denote a set of switching times. Given a starting solution of the type shown above, the next stage is to specify the genetic operators and the fitness function. The main genetic operators are: (i) mutation, (ii) crossover and (iii) reproduction. Mutation introduces a degree of randomness to help the search to find global solutions without getting trapped in a local minimum. Crossover is defined as the mating of two individuals (the parents) to produce two offspring (the children); however, single-child crossover is also possible. The process may be considered analogous to the division of a mother cell to produce two daughter cells. As with cellular divisions, genetic trails are passed on from the parents to the children during crossover, thereby maintaining the rationale of the search and preventing it from going astray. Reproduction refers to the creation of child genomes from their parents through crossover. The overall scheme is summarized as a block diagram in Fig. 1; as seen there, mutations are continued until a genome of the prescribed fitness is obtained.

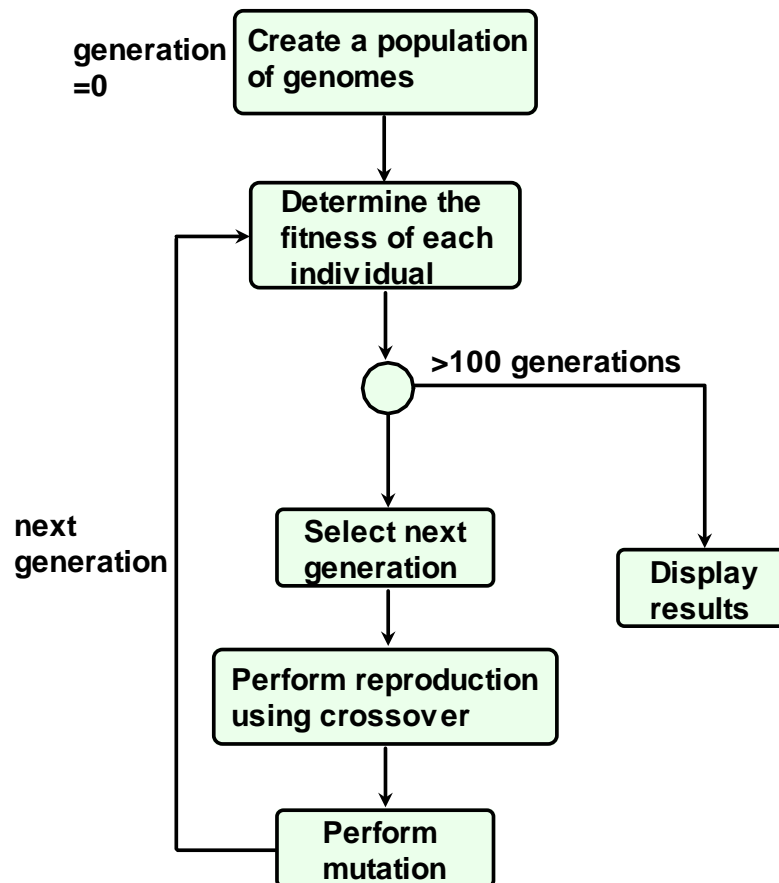


Fig. 1 Flow sheet of the calculation procedure by a genetic algorithm (GA)
 Redrawn from http://www.edc.ncl.ac.uk/asseta/hilite_graphics/rhjan07g01.png

In this application, a uniform mutation was employed for the integral part of each genome and non-uniform mutation for the real parts. Previous studies [18, 19, 22] have suggested arithmetic crossover as being simple logical and effective; here a linear combination of two vector parents produces to vector children. As a GA progresses, the starting population of solutions gets reduced stage by stage until the optimum is reached. This is done by replacing ‘bad’ genomes by ‘good’ ones. To do this, the merit of a genome is quantified by a fitness function. Since the objective here is to maximize PHB concentration at the final time, t_f , the fitness function is simply this performance index as defined in Eq. (8). The replacement strategy also incorporated elitism to increase the rate of convergence [18, 19], this was done by transferring the two best genomes of each generation and each feed stream (glucose and $(\text{NH}_4)_2\text{SO}_4$) to the next generation. Elitism has an effect similar to the momentum of a neuron in a neural network.

The stochastic remainder roulette wheel (SRRW) method was used as the reproduction operator. Owing to the complexity of the fermentation model, the fitness function may have a number of local optima at each stage; the SRRW method ensures that the global optimum is reached quickly at each stage [34, 35].

Results and discussion

The superiority of co-cultures over single cultures of *R. eutropha* in fed-batch fermentations has been shown through many investigations [23, 24, 36, 37]. Hence comparisons with single

culture performance are omitted here. It may, however, be mentioned that co-cultures generate more of the product in both fully dispersed and optimally dispersed bioreactors [32, 37]. Since two organisms with different growth characteristics and different metabolic requirements are involved in the co-culture studied here, the final concentrations of both, as well as the total concentration of biomass, are shown in Fig. 2. The bar charts labeled ‘This work’ are for a fermentation optimized by using a genetic algorithm for a bioreactor operating at a finite dispersion of $Pe = 20$. The results obtained by Tohyama and coworkers [24] are shown along side for comparison. They pertain to a fully dispersed bioreactor ($Pe \rightarrow 0$) optimized by Pontryagin’s maximum principle.

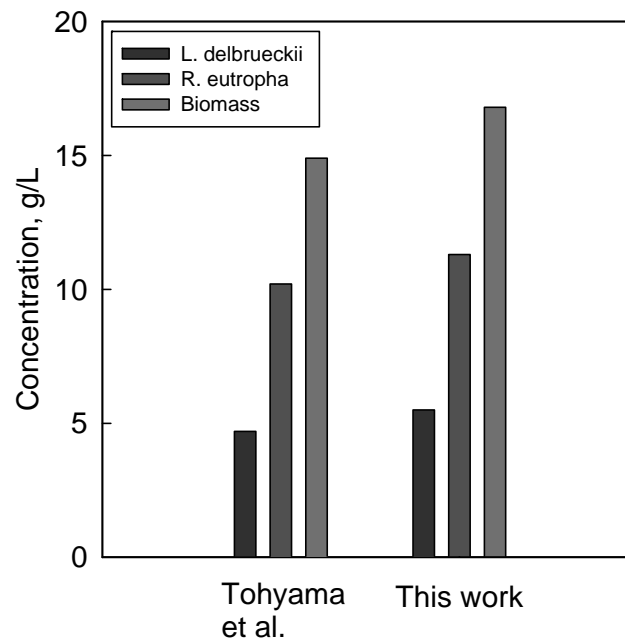


Fig. 2 Final (30 h) concentrations of *L. delbrueckii*, *R. eutropha* and biomass obtained by Tohyama et al. [24] and in the present work

Figs. 3 and 4 present similar comparisons for the final concentration of PHB (in both g/L broth) and g/(g biomass)) and the volume of glucose and $(\text{NH}_4)_2\text{SO}_4$ utilized. The concentrations of the substrates fed in all simulations were the same as in Tohyama et al. [24], i.e. $3\text{g } (\text{NH}_4)_2\text{SO}_4 \cdot \text{l}^{-1}$ and $80\text{g glucose} \cdot \text{l}^{-1}$ in the inlet streams. It is evident from all three figures that co-culture performance through genetic optimization of an optimally dispersed bioreactor is significantly better than by analytic optimization of a fully dispersed culture.

Since optimum dispersion favors product formation regardless of the optimization method, the improved performances in Figs. 2-4 reflect the more efficient feed strategies generated by GA than by analytic methods. The magnitudes of the improvements becomes more explicit in Fig. 5, where PHB concentration is seen to increase by 22.2% per liter of the broth and 8.5% per gram of biomass. The total biomass concentration itself rises by 12.8%.

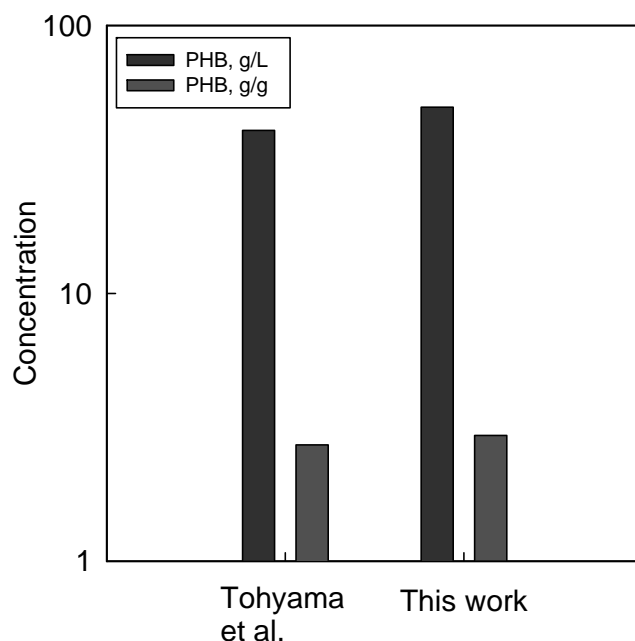


Fig. 3 Comparison of the final volumetric ($\text{g}\cdot\text{L}^{-1}$) and gravimetric ($\text{g}\cdot\text{g}^{-1}$) concentrations of PHB obtained by Tohyama et al. [24] and in the present work

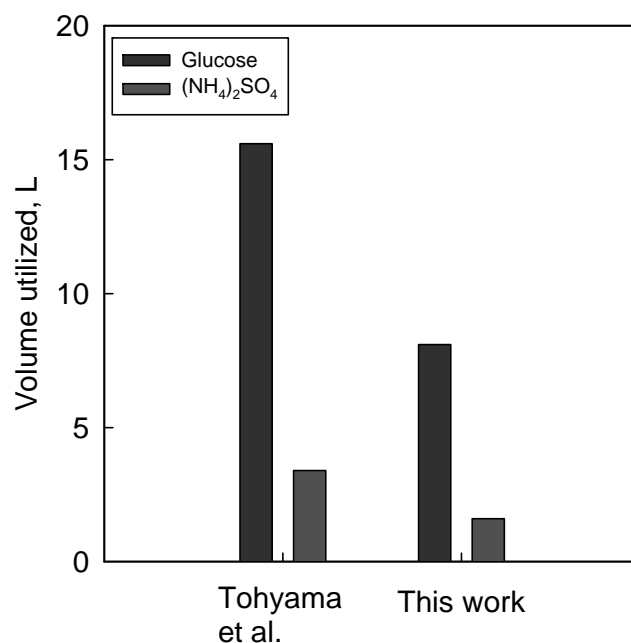


Fig. 4 Comparison of the total consumption of glucose and (NH₄)₂SO₄ in Tohyama et al.'s [24] work and in the present study

The significance of these observations may be understood from the fact that although co-cultures yield higher concentrations of PHB per unit mass of cells, the volumetric concentrations have often been lower than for pure cultures because of poorer growth of mixed cultures [3, 24, 36]. A prime reason for the lower growth of mixed cultures lies in the different, and often contradictory, oxygen requirements of the constituents. Maintaining an

optimum level of dispersion alleviates this problem, and the GA helps by adjusting the inflow rates of the carbon and nitrogen substrates such that the DO concentration is never too high or too low. Tohyama et al. [24] varied the DO concentration step-wise between 0 ppm (to favor *L. delbrueckii*) and 3 ppm (for *R. eutropha*). They tried both equal and unequal intervals for the two levels but there was no significant difference in the PHB output. It is therefore possible that since both organisms are present simultaneously in the fermentation broth, neither complete cut-off nor an overwhelmingly high supply of oxygen (from the perspective of *L. delbrueckii*) is desirable. GA-optimization avoids both extremes and varies the DO concentration dynamically according to the varying requirements of the components of the mixed culture. This dynamic variation helps to achieve greater PHB synthesis with reduced consumption of the substrates (Figs. 3 and 4).

The critical effects of DO and dispersion have been discussed in detail by Tohyama et al. [24] and Patnaik [32] respectively. Briefly, the effect of DO is linked to the central role of lactate. Since lactate is produced by *L. delbrueckii*, which is anaerobic, and consumed by *R. eutropha*, which requires DO, control of the DO concentration has a decisive influence on cell growth and PHB synthesis. Additionally, although PHB formation by *R. eutropha* increases with increasing lactate concentration, too high a concentration of lactate depresses cell growth [24] just as excess of glucose does [26]. Hence the concentration of lactate in the fermentation broth should not be allowed to fall too low or rise too high, and this is ensured through DO control. Lactate also has a pivotal role in the metabolism of PHB synthesis, which is outside the scope of this communication but has been described by Tohyama and Shimizu [30] and Tohyama et al. [24]. In fact, Tohyama et al. [24] recommended maintaining the lactate concentration around $\sqrt{K_p K_l}$ for best results.

Any beneficial effect of controlled dispersion might seem anomalous since classical bioreactor theory teaches that infinite dispersion is ideally the best choice. To explain this anomaly, we note that, other than lactate, acetate too is an intermediate in the PHB biosynthesis pathway [6-8]. High concentrations of PHB suppress acetate formation, which is favorable since acetate inhibits the growth of *R. eutropha*. However, extremely fast synthesis of PHB, resulting in very high intra-cellular concentrations, is unfavorable as it exerts a high metabolic stress on the cells, thereby depleting the availability of energy processors for the other cellular functions [27, 38]. Now, in a well-dispersed broth the nitrogen and carbon substrates are freely available to all cells; this favors the formation of acetate (and lactate), whose accumulation inhibits the growth of *R. eutropha*, thus resulting in low volumetric concentrations of PHB even while its intra-cellular concentration is high [3, 7, 27]. An additional problem with high dispersion is that the rapid formation of PHB consumes large quantities of carbon, thereby depriving the cells of sufficient carbon. Then, to meet their metabolic requirements, the cells utilize PHB as the carbon source, thereby degrading the polymer [28, 39]. On the other hand, poor dispersion (i.e. a high value of Pe) restricts the availability of the substrates to the cells and thus reduces PHB formation.

Since dispersion and carbon metabolism are inter-connected, as explained above, it is important to maintain an optimum concentration of carbon in the bioreactor at all times. In fed-batch operation this optimum value may vary with time and it determines how the supply of $(\text{NH}_4)_2\text{SO}_4$ should be regulated. By its anticipatory nature, its adjustability to evolving situations and its accurate portrayal of the fermentation kinetics, a GA method is able to exercise more efficient and intelligent control of carbon and nitrogen supply than conventional analytic or numeric control polices do.

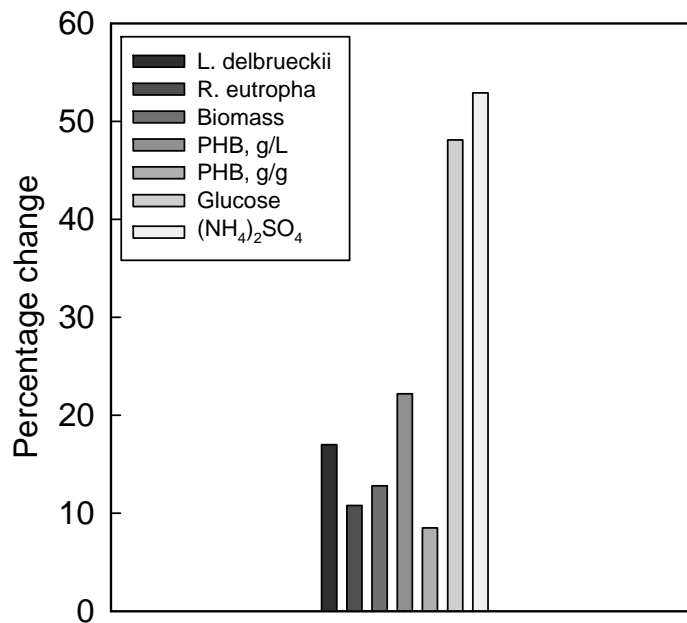


Fig. 5 Percentage change for the concentrations of different components and the consumption of the substrates from Tohyama et al.'s [24] study to the present work

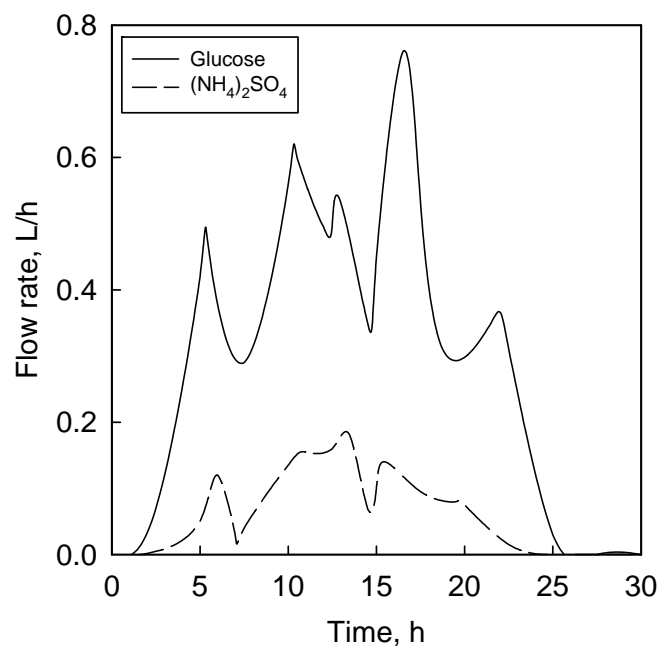


Fig. 6 Genetic algorithmic optimized flow rates of glucose and (NH₄)₂SO₄

The time-dependent GA-optimized flow rates of glucose and (NH₄)₂SO₄ are shown in Fig. 6, and the concentrations of the total biomass and PHB in Figs. 7 and 8.

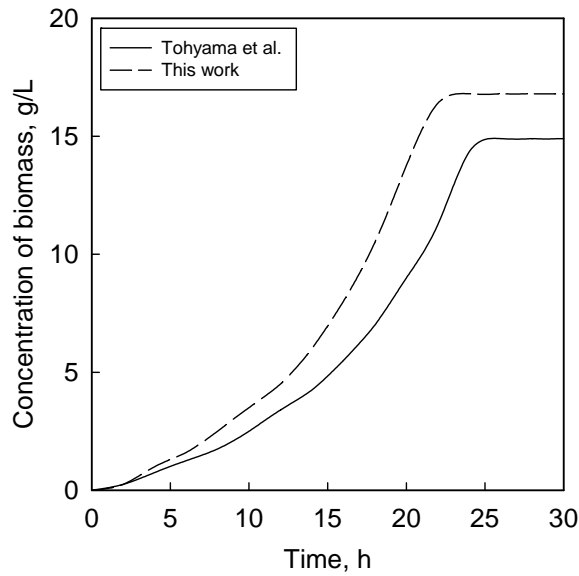


Fig. 7 Increase in the concentration of the total biomass with time for a fully dispersed bioreactor (Tohyama et al. [24]) and an optimally dispersed reactor optimized by a GA

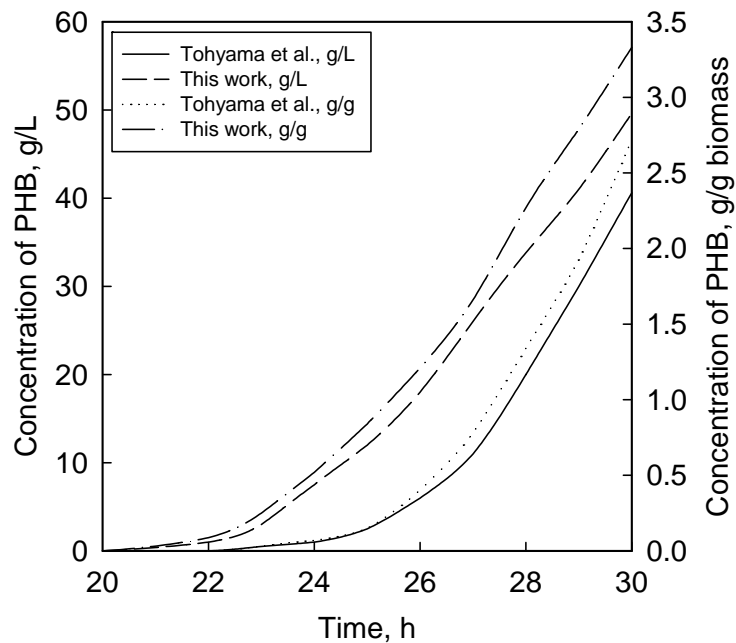


Fig. 8 Increase in the concentration of PHB with time for a fully dispersed bioreactor (Tohyama et al. [24]) and an optimally dispersed reactor optimized by a GA

The flow rates in Fig. 6 are smoother than those obtained by Tohyama et al. [24], thus enabling easier control. Moreover, it is seen from Figs. 3, 4, 7 and 8 that genetic optimization results in lower substrate consumption and higher product (PHB) formation than that achieved by analytically optimized feed rate profiles [24]. While the PHB concentration increases through GA in terms of both reactor volume and biomass quantity, the latter improvements

are moderated by the concomitant increases in total cell mass. Nevertheless, the final PHB concentration per unit cell mass was $2.95 \text{ g}\cdot\text{g}^{-1}$ as compared to $2.72 \text{ g}\cdot\text{g}^{-1}$ reported by Tohyama et al. [24].

Thus, GA is a promising approach for the enhancement of PHB production by a co-culture. Similar improvements for other fermentation systems [14, 15, 17, 21, 33] indicate the general feasibility of GAs for the optimization of fed-batch bioreactors under nonideal conditions.

Conclusions

The use of co-cultures of two or more organisms in place of single cultures for the production of PHB offers a number of advantages. However, the fermentation is also more difficult to model and optimize. This has been explored here for a fed-batch fermentation with *L. delbrueckii* and *R. eutropha*. The former being anaerobic and the latter aerobic, oxygen supply had to be alternated between low and high values.

To mimic a large nonideal bioreactor, dispersion in the fermentation broth was maintained at $Pe = 20$, which had been determined earlier [32] to be the optimum value. Since laboratory-scale models and analytic optimization are inappropriate for nonideal fermentations, the process was optimized by a genetic algorithm (GA). The GA used differed from conventional applications in that the feed rates of two main substrates – glucose and $(\text{NH}_4)_2\text{SO}_4$ – had to be optimized as the fermentation progressed.

Optimization of an optimally dispersed bioreactor by a GA resulted in 22.2% increase in PHB concentration per liter and 8.5% increase per unit biomass. The improvement in terms of biomass was smaller because the biomass itself increased by 12.8%, a significant departure from most mixed culture fermentations, where the increased biosynthesis of PHB has been offset by smaller growth rates of the cells.

Apart from the contrasting DO requirements of the component cultures, the intermediate lactate and DO itself have a controlling influence on the fermentation. The rate of PHB formation increases up to a critical concentration of lactate and decreases thereafter. Thus it is important to maintain the critical concentration at all times, which requires accurate monitoring and control of the two feed streams. In view of the complexity of the system, GA achieves this in a more efficient and facile manner than analytic optimization.

DO controls the net rate of lactate formation [24, 30]. The supply of oxygen or air is therefore interconnected with those of $(\text{NH}_4)_2\text{SO}_4$ and glucose. In addition, the fermentation is highly sensitive to fluctuations in the DO concentration when Pe is near the optimum value [40]. Hence, while optimum dispersion enhances PHB, production, it also makes the process more sensitive to disturbances. These opposing effects may be overcome either by more stringent control or by operating at a somewhat sub-optimal Pe ; the latter method to maintain reactor stability and prevent run-away excursions has been favored since it is cheaper and more convenient.

Appendix

Tohyama et al. [24] proposed the kinetic model presented below for a co-culture of *L. delbrueckii* and *R. eutropha* grown on glucose and $(\text{NH}_4)_2\text{SO}_4$ as the primary substrates.

The rate of growth of *L. delbrueckii* is

$$r_1 = \frac{dX_1}{dt} = \mu_1(S, P, O)X_1 \quad (21)$$

and that of *R. eutropha* follows a similar form

$$r_2 = \frac{dX_2}{dt} = \mu_2(N, P, O)X_2 \quad (22)$$

Glucose is utilized by *L. delbrueckii* at the rate

$$r_s = \frac{dS}{dt} = -v_1(S, P, O)X_1 \quad (23)$$

Since lactate is produced by *L. delbrueckii* and consumed by *R. eutropha*, its net rate of change is

$$r_p = \frac{dP}{dt} = \sigma_1(S, P, O)X_1 - v_2(N, P, O)X_2 \quad (24)$$

In Eqs. (21) – (24), μ_1, μ_2, v_1, v_2 and σ_1 are specific rates which follow the equations given below.

$$\mu_1(S, P, O) = \frac{\mu_{m1}(O)S}{K_S + S} \left(1 - \frac{P}{P_m}\right)^n \quad (25)$$

$$\mu_2(N, P, O) = \left(\frac{\mu_{m2}(O)P}{K_p + P + P^2/K_I}\right) \left(\frac{N}{K_N + N}\right) \quad (26)$$

$$v_1(S, P, O) = \frac{\mu_1(S, P, O)}{Y_{X1/S}(O)} + \frac{\sigma_1(S, P, O)}{Y_{P/S}(O)} \quad (27)$$

$$v_2(N, P, O) = \frac{\mu_2(N, P, O)}{Y_{X2/P}(O)} \quad (28)$$

$$\sigma_1(S, P, O) = \alpha\mu_1(S, P, O) + \beta(S, O) \quad (29)$$

It may be noted that the specific rate of lactate formation, Eq. (29), has a constitutive component β , and a growth related component $\alpha\mu_1$. This form reflects the fact that glucose is utilized by *L. delbrueckii* for growth as well as lactate synthesis. The constitutive rate has the form

$$\beta(S, O) = \frac{\beta_m(O)S}{K_S + S} \quad (30)$$

The rates of $(\text{NH}_4)_2\text{SO}_4$ consumption and PHB formation follow equations similar to those for glucose and the two cultures.

$$r_N = \frac{dN}{dt} = -v_3(N, P, O)X_2 \quad (31)$$

$$r_Q = \frac{dQ}{dt} = \sigma_2(N)X_2 \quad (32)$$

The two specific rates here are described by

$$\sigma_2(N) = \frac{q_m k_N}{k_N + N} \quad (33)$$

$$\text{and } v_3(N, P, O) = \frac{\mu_2(N, P, O)}{Y_{X_2/N}(O)} \quad (34)$$

Tohyama et al. [24] provided an important clarification about Eq. (31). This equation may seem incorrect since it implies that $(\text{NH}_4)_2\text{SO}_4$ is consumed only by *R. eutropha* and not by *L. delbrueckii*. However, Eq. (31) is a practical approximation that reflects their observation that changes in ammonium concentration during the cultivation of *L. delbrueckii* were small compared to changes during the cultivation period of *R. eutropha*. This observation is also contained in Eq. (15), which does not have a nitrogen term for μ_1 . In a similar manner, Eqs. (16) and (33) express the observations that cell growth increased with ammonium concentration whereas that of PHB decreased.

The equations shown above underline the critical role of DO in addition to that of lactate. It affects not only the specific rates of many components but also some of the kinetic parameters. Tohyama et al. [24] proposed the following equations for these parameters.

$$\mu_{m1} = a_1 \exp(-a_2 O) + a_3 \quad (35)$$

$$Y_{P/S} = b_1 \exp(-b_2 O) + b_3 \quad (36)$$

$$\beta_m = c_1 \exp(-c_2 O) + c_3 \quad (37)$$

$$\mu_{m2} = d_1 \exp(-d_2 O) + d_3 \quad (38)$$

$$Y_{X_2/P} = f_1 \exp(-f_2 O) + f_3 \quad (39)$$

$$Y_{Q/P} = g_1 \exp(-g_2 O) + g_3 \quad (40)$$

The values of all the parameters in Eqs. (15)-(40) are listed in Table 1. Together with Eqs. (11)-(14), they define the kinetics without flow terms. To determine the performance of a fed-batch bioreactor with finite dispersion, Eqs. (11)-(14) are inserted into the appropriate mass balances; for a general concentration vector \bar{y} , the dispersed-affected model has the form [41]:

$$\frac{1}{D} \frac{\partial \bar{y}}{\partial t} = \frac{1}{Pe} \frac{\partial^2 \bar{y}}{\partial z^2} - \frac{\partial \bar{y}}{\partial z} \pm \frac{1}{d} \bar{r} \quad (13)$$

For the last term of Eq. (41), a positive sign is used for the concentrations of PHB, *L. delbrueckii* and *R. eutropha*, and a negative sign for the substrates. This set of equations is solved under the following initial and boundary conditions:

$$t = 0 \quad : \quad \bar{y} = \bar{y}_0 \quad \forall z \quad (\text{homogeneity}) \quad (14)$$

$$z = 0 \quad : \quad \frac{\partial \bar{y}}{\partial z} = 0 \quad (\text{symmetry}) \quad (15)$$

$$z = R \quad : \quad \frac{\partial \bar{y}}{\partial z} = 0 \quad (\text{no outflow}) \quad (16)$$

Table 1. List of parameter values and initial conditions [24]

Variable	Units	Value	Variable	Units	Value
α	-	1.2300	Q_0	$\text{g}\cdot\text{l}^{-1}$	0.0000
β_m	h^{-1}	1.8000	S_0	$\text{g}\cdot\text{l}^{-1}$	10.0000
μ_{m1}	h^{-1}	0.3750	S_f	$\text{g}\cdot\text{l}^{-1}$	10.0000
μ_{m2}	h^{-1}	0.7340	a_1	h^{-1}	0.1605
K_I	$\text{g}\cdot\text{l}^{-1}$	2.5000	a_2	$(\text{ppm})^{-1}$	1.4967
k_N	$\text{g}\cdot\text{l}^{-1}$	0.0500	a_3	h^{-1}	0.3395
K_N	$\text{g}\cdot\text{l}^{-1}$	0.1460	b_1	$\text{g}\cdot\text{g}^{-1}$	0.2451
K_P	$\text{g}\cdot\text{l}^{-1}$	6.0000	b_2	$(\text{ppm})^{-1}$	3.5840
K_S	$\text{g}\cdot\text{l}^{-1}$	35.8000	b_3	$\text{g}\cdot\text{g}^{-1}$	0.6909
n	-	1.0000	c_1	h^{-1}	3.3309
P_m	$\text{g}\cdot\text{l}^{-1}$	42.9000	c_2	$(\text{ppm})^{-1}$	3.2574
q_m	h^{-1}	0.6870	c_3	h^{-1}	1.6691
$Y_{P/S}$	$\text{g}\cdot\text{g}^{-1}$	0.6980	d_1	h^{-1}	-8.2410
$Y_{X2/N}$	$\text{g}\cdot\text{g}^{-1}$	2.4100	d_2	$(\text{ppm})^{-1}$	6.5279
$Y_{X2/P}$	$\text{g}\cdot\text{g}^{-1}$	0.2040	d_3	h^{-1}	0.7469
$Y_{X1/S}$	$\text{g}\cdot\text{g}^{-1}$	1.0000	f_1	$\text{g}\cdot\text{g}^{-1}$	2.3600
X_{10}	$\text{g}\cdot\text{l}^{-1}$	0.5000	f_2	$(\text{ppm})^{-1}$	5.2653
X_{20}	$\text{g}\cdot\text{l}^{-1}$	0.0550	f_3	$\text{g}\cdot\text{g}^{-1}$	0.1909
P_0	$\text{g}\cdot\text{l}^{-1}$	0.0000	g_1	$\text{g}\cdot\text{g}^{-1}$	0.7772
N_0	$\text{g}\cdot\text{l}^{-1}$	0.4000	g_2	$(\text{ppm})^{-1}$	3.3097
N_f	$\text{g}\cdot\text{l}^{-1}$	0.4000	g_3	$\text{g}\cdot\text{g}^{-1}$	0.0643

Nomenclature

D	dilution rate, h^{-1}	n	empirical exponent, -
D_e	effective dispersion coefficient, $\text{cm}^2\cdot\text{h}^{-1}$	N	concentration of ammonium sulfate, $\text{g}\cdot\text{l}^{-1}$
F_N	inflow rate of ammonium sulfate, $\text{l}\cdot\text{h}^{-1}$	N_0	feed concentration of ammonium sulfate, $\text{g}\cdot\text{l}^{-1}$
$F_{N, \min}$	lower limit of F_N , $\text{l}\cdot\text{h}^{-1}$	O	concentration of dissolved oxygen, ppm
$F_{N, \max}$	upper limit of F_N , $\text{l}\cdot\text{h}^{-1}$	P	concentration of lactate, $\text{g}\cdot\text{l}^{-1}$
F_S	inflow rate of glucose, $\text{l}\cdot\text{h}^{-1}$	P_m	limiting concentration of lactate, $\text{g}\cdot\text{l}^{-1}$
$F_{S, \min}$	lower limit of F_S , $\text{l}\cdot\text{h}^{-1}$	Pe	Peclet number, -
$F_{S, \max}$	upper limit of F_S , $\text{l}\cdot\text{h}^{-1}$	Q	concentration of PHB, $\text{g}\cdot\text{l}^{-1}$
F_N^*	singular feed rate of $(\text{NH}_4)_2\text{SO}_4$, $\text{l}\cdot\text{h}^{-1}$	q_m	maximum specific PHB production rate, h^{-1}
F_S^*	singular feed rate of glucose, $\text{l}\cdot\text{h}^{-1}$	r_1	rate of growth of <i>L. delbrueckii</i> , $\text{g}\cdot\text{l}^{-1}\cdot\text{h}^{-1}$
ΔF_{NC}	correction term for F_N , $\text{l}\cdot\text{h}^{-1}$	r_2	rate of growth of <i>R. eutropha</i> , $\text{g}\cdot\text{l}^{-1}\cdot\text{h}^{-1}$
ΔF_{SC}	correction term for F_S^* , $\text{l}\cdot\text{h}^{-1}$	r_N	rate of consumption of $(\text{NH}_4)_2\text{SO}_4$, $\text{g}\cdot\text{l}^{-1}\cdot\text{h}^{-1}$
K_i	inhibition constant for μ_2 , $\text{g}\cdot\text{l}^{-1}$	r_P	net rate of formation of lactate, $\text{g}\cdot\text{l}^{-1}\cdot\text{h}^{-1}$
k_N	reaction rate constant for PHB, $\text{g}\cdot\text{l}^{-1}$	r_Q	rate of formation of PHB, $\text{g}\cdot\text{l}^{-1}\cdot\text{h}^{-1}$
K_N	Monod constant for μ_2 with respect to ammonium, $\text{g}\cdot\text{l}^{-1}$	r_S	rate of consumption of glucose, $\text{g}\cdot\text{l}^{-1}\cdot\text{h}^{-1}$
K_P	Monod constant for μ_2 with respect to lactate, $\text{g}\cdot\text{l}^{-1}$	R	radius of the bioreactor, cm
K_S	Monod constant for μ_1 , $\text{g}\cdot\text{l}^{-1}$	S	concentration of glucose, $\text{g}\cdot\text{l}^{-1}$
L	characteristic dimension of bioreactor, cm	S_0	feed concentration of glucose, $\text{g}\cdot\text{l}^{-1}$
		t	elapsed time, h

t_f	final time, h	<i>Greek letters</i>	
u	mean velocity of fluid in bioreactor, $\text{cm}\cdot\text{h}^{-1}$	α	empirical constant, –
V	volume of the broth in the bioreactor, l	β	constitutive component of σ_1 , h^{-1}
V_{max}	maximum value of V , l	β_m	maximum value of β , h^{-1}
X_1	concentration of <i>L. delbrueckii</i> , $\text{g}\cdot\text{l}^{-1}$	μ_1	specific rate of growth of <i>L. delbrueckii</i> , h^{-1}
X_2	concentration of <i>R. eutropha</i> , $\text{g}\cdot\text{l}^{-1}$	μ_2	specific rate of growth of <i>R. eutropha</i> , h^{-1}
$Y_{Q/P}$	yield coefficient for PHB with respect to lactate, $\text{g}\cdot\text{g}^{-1}$	μ_{m1}	maximum value of μ_1 , h^{-1}
$Y_{P/S}$	yield coefficient for lactate with respect to glucose, $\text{g}\cdot\text{g}^{-1}$	μ_{m2}	maximum value of μ_2 , h^{-1}
$Y_{X1/S}$	yield coefficient for <i>L. delbrueckii</i> with respect to glucose, $\text{g}\cdot\text{g}^{-1}$	ν_1	specific rate of consumption of glucose, h^{-1}
$Y_{X2/N}$	yield coefficient for <i>R. eutropha</i> with respect to ammonium, $\text{g}\cdot\text{g}^{-1}$	ν_2	specific rate of consumption of lactate by <i>R. eutropha</i> , h^{-1}
$Y_{X2/P}$	yield coefficient for <i>R. eutropha</i> with respect to lactate, $\text{g}\cdot\text{g}^{-1}$	ν_3	specific rate of consumption of ammonium sulfate, h^{-1}
z	dimensionless distance along radius of bioreactor, –	σ_1	specific rate of production of lactate by <i>L. delbrueckii</i> , h^{-1}
		σ_2	specific rate of formation of PHB, h^{-1}

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