Appl Microbiol Biotechnol (1989) 30:11–17

Applied and Microbiology Biotechnology

Substrate inhibition kinetics for microbial growth and synthesis of poly-β-hydroxybutyric acid by *Alcaligenes eutrophus* ATCC 17697

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Summary. The specific growth rate of *Alcaligenes eutrophus* ATCC 17697 was observed to depend upon the ratio of ammonium sulfate (nitrogen) and fructose (carbon) concentration used for production of poly-β-hydroxybutyric acid (PHB). The growth rate was stimulated at low concentration ratios and inhibited at higher ratios. A mathematical expression was then proposed to fit the substrate inhibition kinetics:

$$\mu = \mu_m \frac{S}{K_s + S}\left[1 - \left(\frac{S}{S_m}\right)^n\right]$$

Together with the Luedeking-Piret product formation equation, the proposed model describes the experimental data as well as literature data, for substrate consumption, growth of *A. eutrophus*, and poly-β-hydroxybutyric acid formation.

Introduction

Under certain unbalanced growth conditions, several microorganisms are known to synthesize poly-β-hydroxybutyric acid (PHB), a biodegradable intracellular polymer (Byrom 1987). PHB is a polyester polymer with potential applications as biodegradable thermoplastic and surgical materials (Senior 1984). This polymer can be formed into films, fibers, sheets or molded into shapes and bottles (Byrom 1987). Various medical applications of PHB and its copolymers are under development by several companies using the PHB product supplied by Marlboro, a subsidiary of Imperial Chemical Industries (ICI).

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Alcaligenes eutrophus, presently used by ICI for PHB production, synthesizes this polymer from a variety of carbon sources under certain nutrient limiting conditions. The ICI process employs a glucose-salt medium to culture *A. eutrophus* in a fed-batch mode.

Mathematical models are necessary for the design, scale-up, optimal control and economic analysis of biopolymer fermentation processes. Consequently, development of kinetic models for describing biopolymer processes has been attempted by several workers. To date, literature models have employed logistic (Ollis 1983) or modified logistic equations to quantify the biomass accumulation rate. Such models were adequate in representing the data of extracellular polymers such as xanthan and pullulan. However, they were not applicable for the description of PHB fermentation processes (Mulchandani et al. 1988; Mulchandani and Luong 1988).

The biomass growth rate in the logistic and modified logistic models is independent of the growth limiting substrate. Their inability to represent the kinetics of PHB fermentation may be due to an absence of relationship between the growth rate and the limiting substrate concentration. Also, recent data on the PHB fermentation using *Protomonas extorquens* indicate a likelihood of substrate inhibition on the growth of the organism (Suzuki et al. 1986). Otherwise, there are no data available on this subject.

The main objective of this study was to develop the kinetic data for describing the inhibitory effect of substrate on the growth of *A. eutrophus*. A kinetic model was then proposed to represent the experimental data. The results of the process simulation derived from the proposed model using the experimental data of this study and the literature were compared and discussed in detail.

Materials and methods

Microorganism and culture medium. *Alcaligenes eutrophus* ATCC 17697 was maintained on nutrient agar slants at 4° C. Inocula were prepared by cultivating the cells for 24 h at 30° C and 160 rpm in a 500 ml shake flask containing 75 ml of the mineral salt medium. The culture medium contained (in % weight/volume):

KH_2PO_4 0.2; Na_2HPO_4 0.06; $MgSO_4 \cdot 7H_2O$ 0.02; $CaCl_2$ 0.002; $ZnSO_4 \cdot 7H_2O$ 0.0013; $FeSO_4 \cdot 7H_2O$ 0.00002; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.00003; H_3BO_3 0.00003; yeast extract 0.1; $(NH_4)_2SO_4$ 0.2 and fructose 4. Fructose, yeast extract, and salt solutions were sterilized separately and then reconstituted aseptically at room temperature (23–25° C). The pH of the medium was adjusted to 7.0 by 3M NaOH solution.

Culture conditions. The bacterium was grown batchwise using 300 ml Nephelo culture flasks equipped with a side arm (Bellco Glass Inc., Vineland, NJ, USA) or a 3.5 l bench-sale fermentor (Chemap AG, Volkestwil, Switzerland).

For growing *A. eutrophus* in Nephelo culture flasks, 50 ml of culture medium containing 0.5% fructose and 0–0.13% (w/v) $(NH_4)_2SO_4$ was inoculated with 2% of inoculum and incubated at 30° C and 160 rpm on a rotary shaker. $O.D_{600}$ was measured periodically to determine specific growth rates. The experiment was performed in triplicate.

The 3.5 l laboratory fermentor was operated with 2.5 l culture medium. The culture broth was agitated at 600 rpm by two six-bladed turbine impellers. The temperature was controlled at 30 ± 1.5° C and oxygen supplied by sparging an air stream at a flow rate of 4.5 l/min (1.8 vvm). The pH was automatically controlled at 7.0 ± 0.2 by adding either 3 M NaOH or 1.5 M H_2SO_4. Samples were collected at regular intervals for determination of cell dry weight, $(NH_4)_2SO_4$, fructose, cellular protein content, and poly-β-hydroxybutyrate (PHB).

Analytical procedures

Cell dry weight. 5 ml of the cell containing broth was centrifuged for 15 min at 3000 *g*-force in a centrifuge, model Centra 4 (International Equipment Co., Needham Heights, Mass., USA). The pellet was washed twice with distilled water and transferred to a preweighed aluminium weighing dish. The cells were dried to a constant weight at 90° C under slight vacuum.

Cellular protein. Cell pellet recovered from 5 ml of culture broth was washed twice with distilled water, resuspended in 5 ml of 1 N NaOH and heated for 10 min at 90° C to solubilize the protein. The protein concentration was determined by the Lowry's method (Lowry et al. 1951) using 1% bovine serum albumin as an external standard.

PHB concentration. Intracellular PHB was determined by the gas chromatographic method developed by Braunegg et al. (1978) using benzoic acid as an internal standard.

Fructose concentration. Fructose concentration was estimated by YSI Model 27 (YSI Incorporated, Yellow Springs, Ohio, USA) Industrial Analzyer. The polarographic electrode was used together with an immobilized galactose oxidase enzyme membrane.

Ammonium sulfate concentration. $(NH_4)_2SO_4$ concentration in the supernatant broth was measured by an ammonia electrode (Orion Research Inc., Cambridge, MA, USA).

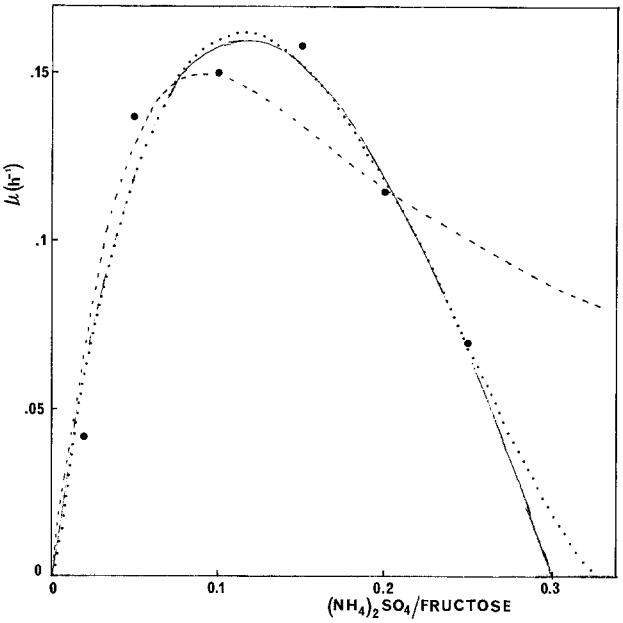

Fig. 1. Effect of the ratio of ammonium sulfate to fructose concentration on *A. eutrophus* growth: (●) experimental data; (· · · · ·) simulation using Eq. (3); (– – – –) simulation using Eq. (4); (——) simulation using Eq. (5)

Numerical calculations. Numerical calculations for model evaluation and computer simulation were performed using a 11/750 VAX computer at the Biotechnology Research Institute, Montreal, in an interactive mode. All the programs were written in FORTRAN language and compiled with a VAX FORTRAN compiler.

Results and discussion

Values of the maximum specific growth rate coefficient, μ, were determined for each initial ammonium sulfate to fructose concentration ratio by plotting ln X vs. time (figure not shown).

Figure 1 shows the effect of the ratio of ammonium sulfate to fructose concentration in the culture medium on the specific growth rate of *A. eutrophus*. The data were generated by varying the ammonium sulfate concentration from 0 to 1.3 g/l while fructose was kept constant at 5 g/l. The relationship is one of conventional substrate inhibition kinetics, where the growth is stimulated at low concentrations of ammonium sulfate and inhibited at high concentrations. The maximal value of the specific growth rate ($\mu = 0.158\ h^{-1}$) was achieved when the ammonium sulfate to fructose ratio was in the range of 0.12–0.16, corresponding to ammonium sulfate concentration of 0.6–0.8 g/l.

In order to validate the relationship between the specific growth rate and the $(NH_4)_2SO_4$ to fructose concentration ratio two additional ex-

periments were conducted. In these experiments the concentration of fructose was 20 g/l and 40 g/l, while $(NH_4)_2SO_4$ was kept constant at 2 g/l. Under these conditions (concentration ratio of 0.05 and 0.1) the maximum specific growth rates were determined to be 0.14 h^{-1} and 0.15 h^{-1}, respectively. These results compared favorably with previously determined values for same concentration ratios (Fig. 1).

Figure 2 depicts the dynamics of the fermentation of *A. eutrophus* grown on 40 g/l fructose and 1.88 g/l ammonium sulfate as carbon and nitrogen source, respectively. After a lag period of approximately 10 h, the biomass (dry weight) increased slowly to 6.4 g/l after 47 h. In this period, ammonium sulfate was completely consumed, whereas approximately 15 g/l of fructose was metabolized. The synthesis of PHB, the product of interest, commenced after approximately 18 h and then reached a final concentration of 3.78 g/l. The PHB yield based on the amount of fructose consumed was 0.25 g/g. This was significantly higher than the value of 0.07 g/g reported by Ramsay and Cooper (1987). In this study, the whole cell dry weight is considered to consist of two parts, namely, PHB and residual biomass. The residual biomass, a difference between cell dry weight and PHB concentration can be considered as the catalytically active biomass. As shown in Fig.2, after 46 h of fermentation the residual biomass concentration increased to a maximum of 2.8 g/l corresponding to a total depletion of ammonium sulfate. Similarly, the cellular protein, an indicator of the residual biomass synthesis, was also synthesized until the growth limiting substrate, ammonium sulfate, was completely depleted. Throughout the experiment, the dissolved oxygen concentration was always observed to be higher than 80% of saturation level.

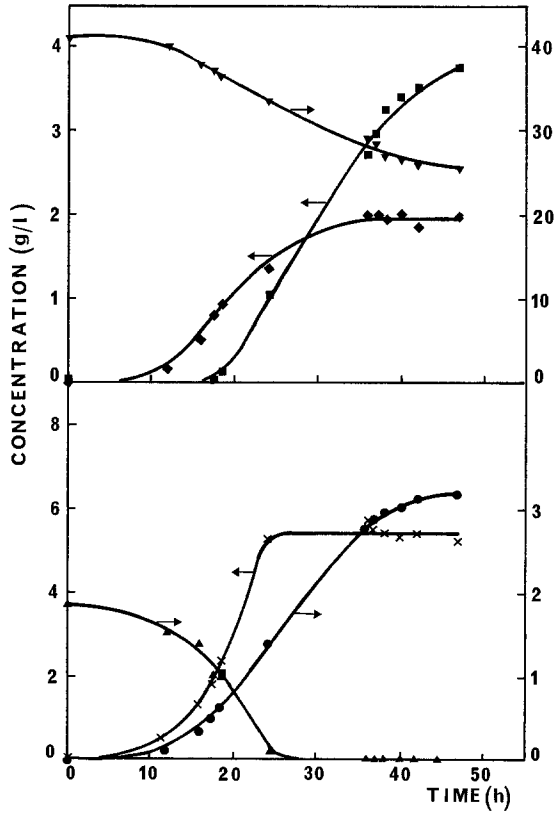

Fig. 2. Dynamics of PHB fermentation. (●) Total dry weight; (×) Residual biomass; (▲) Ammonium sulfate; (▼) Fructose; (■) PHB: (◆) Protein

Mathematical modeling

Based on the experimental data, a batch fermentation kinetics model was developed to represent PHB biosynthesis by *A. eutrophus*.

Rate of cell growth. The exponential growth rate of a microorganism can be expressed by

$$\frac{dX}{dt} = \mu X \tag{1}$$

where the rate of change of cell concentration (dX/dt) is related to the cell concentration by the specific growth rate, μ.

As indicated earlier, the cell components of *A. eutrophus* consist of two main parts — i. e. PHB(P) and the residual biomass (X_R). This residual biomass is the catalytically active component of the cell and it is responsible for the metabolic activity of the cells. The term X in Eq. (1) was therefore replaced by X_R to represent the autocatalytic growth of the cells.

$$\frac{dX_R}{dt} = \mu X_R \tag{2}$$

Figure 1 obviously showed that the specific growth rate was a function of the ratio of ammonium sulfate and fructose concentrations. The relationship appeared to follow conventional substrate inhibition kinetics. In this study, two substrate inhibition models proposed by Andrews (1968) [Eq. (3)] and Luong (1987) [Eq. (4)] were attempted to describe the inhibition kinetics data of *A. eutrophus* (Fig. 1).

$$\mu_i = \mu_m \frac{S}{S + K_s + S^2/K_{si}} \tag{3}$$

$$\mu_i = \mu_m \frac{S}{S + K_s}\left[1 - \frac{S}{S_m}\right]^m \tag{4}$$

In addition, assuming a common mechanism between product and substrate inhibition, the relationship proposed by Luong (1985) to describe product inhibition kinetics was used to correlate substrate inhibition [Eq. (5)].

$$\mu = \mu_m \frac{S}{S + K_s}\left[1 - \left(\frac{S}{S_m}\right)^n\right] \tag{5}$$

The exponents m and n in Eqs. (4) and (5), respectively, are fitting parameters, with no physical significance.

The values of the model parameters, (Table 1), i.e. μ_m, K_s, S_m, m and n, were determined by fitting the substrate inhibition data to Eqs. (3) to (5) using a non-linear regression technique. Figure 1 shows the comparison of the three models with the experimental data. As shown in this figure, Eq. (3) was only able to represent the data reasonably well up to a ammonium sulfate to fructose concentration ratio of 0.2. At higher ratios, the model predictions were significantly higher than the experimental data. Furthermore, the model implies that the cells are capable of growing indefinitely, which is contrary to the observed reality.

Eqs. (4) and (5) were virtually similar in their ability to describe the experimental data. However, a careful observation of the model parameters (Table 1) showed that the value of K_s obtained by fitting these data to Eq. (4) was very high and approached S_m, concentration at which the growth is completely inhibited. The Monod's constant, K_s, is defined as the substrate concentration at which the specific growth rate is half of its maximum value. Therefore, it cannot surpass S_m. Consequently, model equation (5) was selected for representing the rate of biomass accumulation in the PHB synthesis process simulation. Eq. (1) can now be written as

Table 1. Values of the model parameters for substrate inhibition

Models	μ_m [h^{-1}]	K_s	K_{si}	S_m	m	n
Eq. (3)	1.48	0.41	0.02	—	—	—
Eq. (4)	1.08	0.31	—	0.33	1.37	—
Eq. (5)	0.72	0.15	—	0.3	—	1.22

$$\frac{dX_R}{dt} = \mu_m \frac{S}{S + K_s}\left[1 - \left(\frac{S}{S_m}\right)^n\right] X_R \tag{6}$$

Rate of PHB accumulation. Figure 2 indicated that *A. eutrophus* synthesized PHB both in the growth and stationary phases. This phenomenon of growth and non-growth associated synthesis can be represented by the Luedeking-Piret type model (Luedeking and Piret 1959).

$$\frac{dP}{dt} = k_1 \frac{dX_R}{dt} + k_2 X_R \tag{7}$$

Rate of ammonium sulfate consumption. An examination of the experimental data (Fig. 2) indicated that ammonium sulfate was completely consumed in the exponential growth phase and corresponded to an increase in the residual biomass growth rate. The rate of ammonium sulfate consumption therefore can be expressed by

$$-\frac{dS_N}{dt} = k_3 \frac{dX_R}{dt} \tag{8}$$

Rate of fructose utilization. The fructose balance for the PHB biosynthesis process may be written as

$$-\frac{dS_F}{dt} = k_7 \frac{dX_R}{dt} + k_8 \frac{dP}{dt} + k_6 X_R \tag{9}$$

In Eq. (9) the last term represents the substrate consumed to support cell viability, even in absence of growth. These activities include cell mobility, enzyme turnover, osmotic work, nutrient storage and other processes referred to as maintenance functions. Substituting Eq. (7) in Eq. (9) gives

$$-\frac{dS_F}{dt} = (k_7 + k_1 k_8)\frac{dX_R}{dt} + (k_6 + k_8 k_2) X_R \tag{10}$$

or

$$-\frac{dS_F}{dt} = k_4 \frac{dX_R}{dt} + k_5 X_R \tag{11}$$

where

$$k_4 = k_7 + k_1 k_8 \tag{12}$$

and

$$k_5 = k_6 + k_8 k_2 \tag{13}$$

The system of Eqs. (6), (7), (8) and (11) represents a mathematical model of the batch kinetics of PHB biosynthesis by *A. eutrophus* metabolizing ammonium sulfate and fructose.

The proposed model was then used to represent the experimental data obtained in this study. The model parameters $k_1 - k_5$, were first evaluated by solving the model equations and minimizing the weighted residuals of the sum of the squares between the model and the experimental data. These values (Table 2) were then used to simulate the profiles of residual biomass, ammonium sulfate, fructose, and PHB concentrations during the course of the fermentation.

Figure 3 shows that there was good agreement between the simulation results and the experimental data. The proposed model appeared to provide an adequate representation of growth and fermentation kinetics of *A. eutrophus*.

The above calculations for parameter estimation and process simulation were performed for period $t > 10$ h, when the cells enter the exponential growth phase.

It was of further interest to extend the applicability of the proposed model to the literature data. In the study of Heinzel and Lafferty (1980), *A. eutrophus* H 16 (ATCC 17699) was grown on a medium containing ammonium sulfate and gaseous substrates consisting of CO_2, H_2 and O_2. These gaseous substrates were present in excess quantity at constant pressure and flow rate. Since no data were available on the rate of consumption of these gases, simulation was only performed for ammonium sulfate. Furthermore, the dynamics of product concentration indicated that the storage

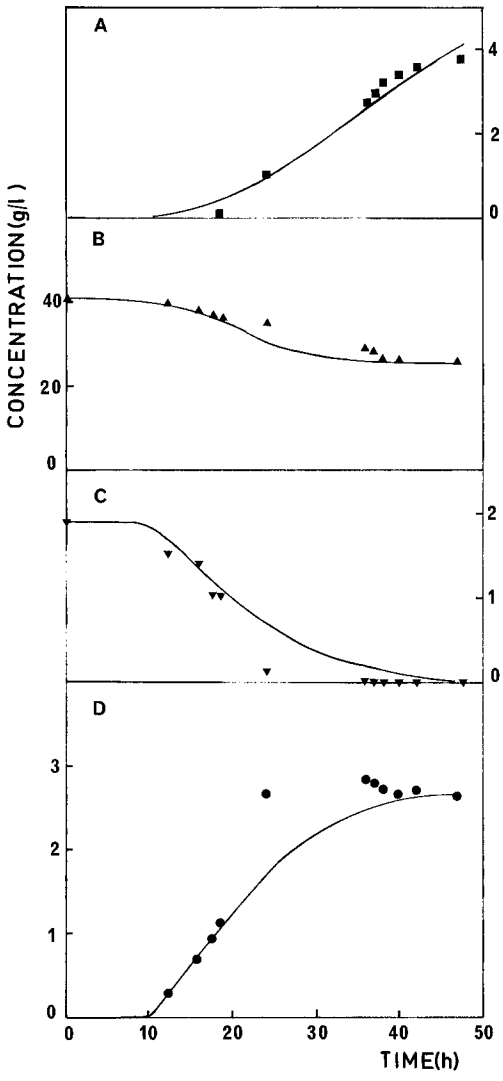

Fig. 3. Comparison of simulation results with the experimental data of this study. **(A)** PHB; **(B)** Fructose; **(C)** Ammonium sulfate; **(D)** Residual biomass

Table 2. Values of the kinetic parameters for process simulation

Kinetic constants	PHB fermentation (this study)	PHB fermentation (Heinzel and Lafferty 1980)
μ_m	0.72 h^{-1}	0.3 h^{-1}
K_s	0.15	—
K_s^*	—	0.88 g/l
S_m	0.3	—
S_m^*	—	5.47 g/l
n	1.22	1.77
k_1	0.106 g_{prod}/g_{cell}	*N/A*
k_2	0.059 $g_{prod}/g_{cell} \cdot h$	*N/A*
k_3	0.685 g_{sub}/g_{cell}	0.65 g_{sub}/g_{cell}
k_4	5.75 g_{sub}/g_{cell}	*N/A*
k_5	0.001 $g_{sub}/g_{cell} \cdot h$	*N/A*
X_m	*N/A*	4.03
p	*N/A*	0.15 $g_{prod}/g_{cell} \cdot h$
a	*N/A*	0.023 h^{-1}

polymer was synthesized only in the stationary phase with steady decrease in the accumulation rate. In order to account for this behavior Ollis (1983) proposed a modified form for product accumulation rate and therefore Eq. (8) was replaced by

$$\frac{dP}{dt'} = pX_m(1 - at') \tag{14}$$

where t' was measured from the onset of the stationary phase ($= 15.5$ h).

Figure 4 shows that the simulation results using the proposed substrate inhibition model compared favorably with the experimental data for all the process parameters. The logistic and modified

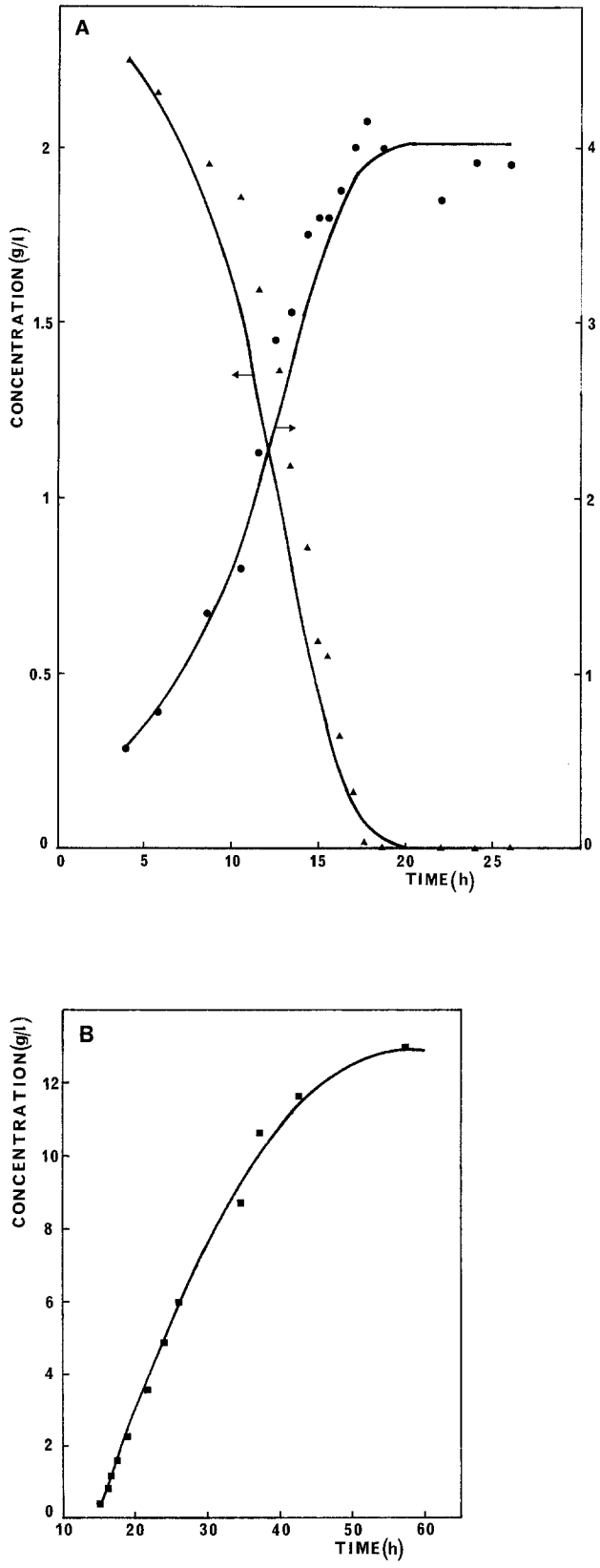

Fig. 4. Comparison of simulation results with experimental data of Heinzel and Lafferty (1980) (●) Residual biomass; (▲) Ammonium sulfate; (■) PHB

logistic models were able to represent only the biomass and PHB concentration data adequately. In view of ammonium sulfate consumption there was a significant deviation between the simulation results and the experimental data (Mulchandani et al. 1988; Luong et al. 1988).

It is apparent from the above that the specific growth rate of *A. eutrophus* is a function of the ratio of ammonium sulfate (nitrogen) to fructose (carbon) concentration. The growth rate of the microorganism is stimulated at low concentration ratios and inhibited at higher ratios. The growth rate model accounts for this phenomenon, and it can be used together with Luedeking-Piret product formation model to describe the kinetic data of batch PHB fermentations by *A. eutrophus*.

Nomenclature

a, constant in Eq. (14) (h^{-1})
k_1, kinetic parameter in Eq. (7) (g_{prod}/g_{cell})
k_2, kinetic parameter in Eq. (7) ($g_{prod}/g_{cell} \cdot h$)
k_3, kinetic parameter in Eq. (8) (g_{sub}/g_{cell})
k_4, kinetic parameter in Eq. (11) (g_{sub}/g_{cell})
k_5, kinetic parameter in Eq. (11) ($g_{sub}/g_{cell} \cdot h$)
k_6, kinetic parameter in Eq. (9) ($g_{sub}/g_{cell} \cdot h$)
k_7, kinetic parameter in Eq. (9) (g_{sub}/g_{cell})
k_8, kinetic parameter in Eq. (9) (g_{sub}/g_{prod})
K_s, saturation constant in Eqs. (3)–(6)
K_s^*, saturation constant (Table 2) (g/l)
K_{si}, inhibition constant in Eq. (3)
m, exponent in Eq. (4)
n, exponent in Eqs. (5) and (6)
p, constant in Eq. (14) ($g_{prod}/g_{cell} \cdot h$)
P, PHB concentration (g/l)
S, ratio of $(NH_4)_2SO_4$ to fructose concentration
S_f, concentration of fructose (g/l)
S_m, ratio of $(NH_4)_2SO_4$ to fructose concentration at which specific growth rate to zero
S_m^*, maximum $(NH_4)_2SO_4$ concentration at which specific growth rate is zero (Table 2) (g/l)
X, total biomass concentration (g/l)
X_R, residual biomass concentration (g/l)
X_m, maximum biomass concentration attained in the stationary phase (g/l)

Greek letters

μ, specific growth rate (h^{-1})
μ_m, maximum specific growth rate (h^{-1})

References

Andrews JF (1968) A mathematical model for the continuous culture of microorganisms utilizing inhibiting substrates. Biotechnol Bioeng 10:707–723

Braunegg G, Sonnleitner B, Lafferty RM (1978) A rapid gas chromatographic method for determination of poly-β-hydroxybutyric acid in microbial biomass. Eur J Appl Microbiol Biotechnol 6:29–37

Byrom D (1987) Polymer synthesis by microorganisms: technology and economics. TIBTECH 5:246–250

Heinzle E, Lafferty RM (1980) A kinetic model for growth and synthesis of poly-β-hydroxybutyric acid (PHB) in *Alcaligenes eutrophus* H 16. Eur J Appl Microbiol Biotechnol 11:8–16

Luedeking R, Piret EL (1959) A kinetic study of the lactic acid fermentation. J Biochem Microbiol Technol Eng 1:393–431

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Luong JHT (1987) Generalization of Monod kinetics for analysis of growth data with substrate inhibition. Biotechnol Bioeng 29:242–248

Luong JHT, Mulchandani A, LeDuy A (1988) Kinetics of biopolymer synthesis: a revisit. Enzyme Microb Technol 10:326–332

Mulchandani A, Luong JHT (1988) Microbial inhibition kinetics revisited. Enzyme Microb Technol (in press)

Mulchandani A, Luong JHT, LeDuy A (1988) Batch kinetics of microbial polysaccharide biosynthesis. Biotechnol Bioeng 32:639–646

Ollis DF (1983) A simple batch fermentation model: theme and variation. Ann NY Acad Sci 413:144–156

Ramsay BA, Cooper DG (1987) Use of *Pseudomonas cepacia* to produce the thermoplastic PHB from organic wastes. 37th. Can Chem Eng Conf Proc 227–279

Senior PJ (1984) Poly-hydroxybutyrate, a specialty polymer of microbial origin. In: Dean ACR, Ellwood DC, Evans CGT (eds) Continuous culture; biotechnology, medicine and the environment. Ellis Horwood Ltd. Cherchester 8:266–271

Suzuki T, Yamane T, Shimizu S (1986) Kinetics and effect of nitrogen source feeding on production of poly-β-hydroxybutyric acid by fed-batch culture. Appl Microbiol Biotechnol 24:366–369

Received May 10, 1988/Accepted August 24, 1988