Poly(β-hydroxybutyric acid) thermoplastic production by *Alcaligenes latus*: Behavior of fed-batch cultures

E. Grothe, Y. Chisti

Abstract Fed-batch culture of *Alcaligenes latus*, ATCC 29713, was investigated for producing the intracellular bioplastic poly(β-hydroxybutyric acid), PHB. Constant rate feeding, exponentially increasing feeding rate, and pH-stat fed batch methods were evaluated. pH-stat fed batch culture reduced or delayed accumulation of the substrate in the broth and led to significantly enhanced PHB productivity relative to the other modes of feeding. Presence of excessive substrate appeared to inhibit PHB synthesis, but not the production of cells. In fed-batch culture, the maximum specific growth rate (0.265 h⁻¹) greatly exceeded the value (0.075 h⁻¹) previously observed in batch culture of the same strain. Similarly, the maximum PHB production rate (up to 1.15 g·l⁻¹·h⁻¹) was nearly 8-fold greater than values observed in batch operations. Fed-batch operation was clearly superior to batch fermentation for producing PHB. A low growth rate was not a prerequisite for PHB accumulation, but a reduced or delayed accumulation of substrate appeared to enhance PHB accumulation. Under the best conditions, PHB concentrations attained were about 18 g·l⁻¹.

List of Symbols

- \(F_T\) mass flow rate of the trace element solution (g·h⁻¹)
- \(m\) maintenance coefficient (–)
- \(N\) concentration of nitrogen source (g·l⁻¹)
- \(P_{m\,x}\) maximum specific product formation rate (g·l⁻¹·h⁻¹)
- \(q_p\) specific product formation rate (g·l⁻¹·h⁻¹)
- \(q_{P_{max}}\) maximum specific product formation rate (g·l⁻¹·h⁻¹)
- \(S\) total amount of substrate in fermenter at time \(t\) (g)
- \(S_0\) mass fraction of the substrate in feed (–)
- \(t\) time (h)
- \(t_0\) x-intercept in Eq. (1) (h)
- \(T\) mass flow rate of the trace element solution (g·h⁻¹)
- \(X_0\) total initial biomass in fermenter (g)
- \(X_t\) total biomass in fermenter at time \(t\) (g)
- \(Y_{P/S}\) product yield coefficient on sucrose (–)
- \(Y_{P/X}\) specific product yield coefficient (–)
- \(Y_{R/N}\) PHB-free biomass yield coefficient on nitrogen source (–)
- \(Y_{R/S}\) PHB-free biomass yield coefficient on carbon source (–)
- \(Y_{R/T}\) PHB-free biomass yield coefficient on trace elements (–)
- \(Y_{X/S}\) biomass yield coefficient on sucrose (–)

Greek symbols

- \(\delta\) mass fraction of PHB in cells (–)
- \(\mu\) specified growth rate (h⁻¹)
- \(\mu_{\text{max}}\) maximum specific growth rate (h⁻¹)

1 Introduction

Microorganisms produce a variety of thermoplastics (Poirier et al., 1995; Kim et al., 1998; Braunegg et al., 1998). Unlike petrochemical-derived polymers, microbial thermoplastics are a renewable resource with important advantages of biodegradability, biocompatibility, and non-toxicity. Despite their relatively high cost, bioplastics are attracting increasing commercial attention (Kim et al., 1998). Microbial poly(β-hydroxybutyric acid), PHB, is especially attractive because its properties are broadly similar to those of some well-established synthetic polymers such as polypropylene (Barham, 1990); hence, PHB or its blends can potentially replace traditional synthetic...
polymers in some applications. PHB is resistant to water and ultraviolet radiation, and impermeable to oxygen. PHB is readily biodegraded in soil. Moreover, processing PHB into articles of commerce does not require new investments in technology; existing equipment, developed originally for processing polyethylene and polypropylene, can be used. Because of its high price – about 15-fold greater than comparable synthetic plastics – applications of PHB are limited to specialist niches. The polymer is potentially useful in slow release drug formulations and biodegradable surgical implants; other uses are in development (Kim et al., 1998).

Low-cost production of PHB requires improved fermentation strategies, inexpensive media, and easier downstream recovery methods (Chisti, 1998; Tamer et al., 1998a, b). This work reports on production of PHB in single-stage fed-batch cultures of the bacterium Alcaligenes latus. In the past a different bacterium, Alcaligenes eutrophus, has been the focus of attention as a producer of PHB, but that microorganism requires an expensive two-stage cultivation (Byrom, 1990; Marchessault et al., 1990). Unlike A. eutrophus, A. latus is a growth-associated producer of PHB; hence, a single-stage fermentation is sufficient (Hrabak, 1992; Hänggi, 1990). Furthermore, A. latus grows readily on sucrose which is less expensive than the glucose-based media that are typically used in A. eutrophus fermentations. Relatively little work has been reported on A. latus (Yamane et al., 1996; Wang and Lee, 1997; Tamer et al., 1998a, b; Grothe et al., 1999) and some of this work focused exclusively on the downstream recovery of PHB (Tamer et al., 1998a, b).

As for any microbial production process (Ejiofor et al., 1996a; Chisti, 1999; Chisti and Moo-Young, 1999), the performance of A. latus culture is susceptible to many influences, including temperature, pH, carbon-to-nitrogen ratio in the feed, concentration of substrates, concentration of trace elements, ionic strength, agitation intensity, and the dissolved oxygen level. Effects of some of these variables have been studied (Yamane et al., 1996; Wang and Lee, 1997; Grothe et al., 1999) and optimal growth and production conditions in shake flask batch culture have been identified in statistically designed experiments (Grothe et al., 1999). Further work is needed in more controlled conditions than are possible in shake flasks. Many examples exist of substantial enhancement in productivity through optimization of fermentation under conditions that are relevant to industrial culture (Chisti and Moo-Young, 1996; Chisti, 1999) and similar work is needed for the A. latus PHB fermentation. Moreover, compared to batch culture, fed-batch operation can enhance yield and productivity by eliminating possible substrate inhibition, or by otherwise modifying metabolic behavior. This work characterizes controlled fed-batch production of PHB using various feeding strategies.

### Table 1. Composition of culture media

<table>
<thead>
<tr>
<th>Component</th>
<th>Composition (g · l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium 1018</td>
</tr>
<tr>
<td>Sucrose</td>
<td>20</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1</td>
</tr>
<tr>
<td>KH₂PO₄</td>
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</tr>
<tr>
<td>Na₂HPO₄</td>
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<tr>
<td>MgSO₄·7H₂O</td>
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<tr>
<td>Trace element solution (ml · l⁻¹)</td>
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</tbody>
</table>

Composition of trace element solution (g · l⁻¹)

<table>
<thead>
<tr>
<th></th>
<th>TES 0</th>
<th>TES 1</th>
<th>TES 2</th>
<th>TES 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Fe(III)citrate</td>
<td>50</td>
<td>60</td>
<td>60</td>
<td>6</td>
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<tr>
<td>CaCl₂·2H₂O</td>
<td>5</td>
<td>10</td>
<td>6.2</td>
<td>10</td>
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<tr>
<td>H₂BO₃</td>
<td>–</td>
<td>0.3</td>
<td>2.8</td>
<td>0.3</td>
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<tr>
<td>CoCl₂·6H₂O</td>
<td>–</td>
<td>0.2</td>
<td>3</td>
<td>0.2</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>–</td>
<td>0.1</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>–</td>
<td>0.03</td>
<td>2</td>
<td>0.03</td>
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<tr>
<td>Na₂MoO₄·2H₂O</td>
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<td>0.5</td>
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<tr>
<td>NiSO₄·7H₂O</td>
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<tr>
<td>CuSO₄·5H₂O</td>
<td>–</td>
<td>0.01</td>
<td>0.4</td>
<td>0.01</td>
</tr>
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</table>

2 Materials and methods

2.1 Microorganism and culture conditions

An intracellular PHB producer, Alcaligenes latus, ATCC 29714 (or DSM 1123), was used throughout. The strain was maintained on agar slants and Petri dishes on the minimal Medium 1018 (Table 1) as noted in the ATCC catalog (Gharda and Rienta, 1995; Grothe et al., 1999). After sufficient growth at 33 °C, the culture was held at 4 °C until needed. Shake flasks (200 ml) containing 50 ml minimal Medium 1018 were inoculated with a loopful of cells. After 3–4 days growth (33 °C, 200 rpm shaker) the 50 ml inoculum attained cloudiness. The inoculum was used directly for the next preculture stage, or held at 4 °C until needed.

A further preculture step was necessary for inoculation of bioreactor. Thus, 5 ml of above described inoculum was transferred to a 2 l shake flask containing Medium 1 (Table 1) with 0.5 ml · l⁻¹ of trace element solution TES 1 instead of the normal 1 ml · l⁻¹ noted in Table 1 (Lafferty and Braunegg, 1990; Ramsay et al., 1990a; Yamane et al.,
Phosphates were dissolved separately to prevent precipitation, and the trace element solution was filter sterilized. The measured pH was 7.2. After 4-days (33 °C, 200 rpm), the entire contents of the flask were used to inoculate 4.5 l of fermentation medium in the bioreactor. Unless otherwise noted, the fermentation medium was identical to that used in preculture. All media were sterilized at 121 °C, 20 min, and cooled to 33 °C culture temperature that had been earlier established as optimal (Grothe et al., 1999). The fermenter was a 15 l (nominal) bottom-stirred device (MBR Bioreactor AG, Switzerland) equipped with pH, temperature, and dissolved oxygen controllers. The 0.2 m diameter vessel was fully baffled (4-baffles). The working volume was 8 l. The starting volume of each fermentation was 5 l. Two six-bladed impellers, both 0.1 m in diameter, were used on the same shaft. The lower impeller was a downward pumping hydrofoil type, whereas the upper impeller was a Rushton turbine (Chisti, 1999; Chisti and Moo-Young, 1999). One of the impellers always remained above the culture level, serving to disperse foam. In addition, Sigma 298 silicon antifoam (Sigma Chemical Co., St. Louis, MO, Catalog no. A 8436) was automatically dispensed in response to a foam sensor. The agitation speed varied over 300–800 rpm in response to a dissolved oxygen controller. The dissolved oxygen concentration generally remained above 15% of air saturation value. The aeration rate was held constant at 1 vvm. The temperature was controlled at that value by addition of aqueous ammonia (20% w/v) solution.

Feeding commenced after an initial batch phase that lasted up to ~35 h. Three different feeding strategies were tested: (i) continuous constant rate feeding; (ii) exponentially increasing feed rate (Eljiofor et al., 1996); and (iii) feeding in response to a pH control signal (pH-stat) as detailed later. Medium 1 and Medium 3 (Table 1) were used for feeding in separate experiments.

2.2 Cell dry mass

Biomass content was determined by gravimetry. Culture samples (10 ml) were centrifuged (15,000 g, 4 min, 4 °C), the supernatant was refrigerated for further analysis, and the cell pellet was washed in deionized water, recovered (15,000 g, 4 min, 4 °C), dried to constant weight (90 °C, 24 h), cooled in a desiccator, and weighed. The biomass yield coefficient on sucrose (Y_{XS}) was calculated as the cell dry weight produced per unit mass of sucrose consumed. All measurements were in duplicate.

2.3 Sucrose

The supernatant (1 ml) from biomass determinations was used in quantifying sucrose by refractive index measurements in an Abbe refractometer (Atago 3T, Japan). The refractometer had been calibrated using dilutions of freshly prepared sucrose-containing uninoculated culture medium (Grothe et al., 1999). In addition, a few of the measurements were verified by HPLC (Millipore, Milford, USA) on a lead sulfate column. Automatic sample injection (Waters 700 Saterlite WISP, 20 µl injection volume) was employed. An external differential refractometer (model R401) was used as the detector. Chromatograms were acquired and integrated with a Baseline 810 Chromatography Workstation. Double deionized water was the eluent (85 °C, 0.6 ml · min⁻¹). The HPLC calibration standards were identical to those used for the refractometer (Grothe et al., 1999).

2.4 Ammonium

A calibrated ammonia sensor electrode (model 8002-8, Electrical Instruments Ltd, Chertsey, U.K.) was used to measure the NH₄⁺ concentration. The measurement range was 50–2,000 mg · l⁻¹ as NH₄⁺. Just prior to measurement, the supernatant (5 ml) from cell dry mass determinations was made alkaline with concentrated sodium hydroxide (1 ml) to convert the dissolved ammonium ion to ammonia (Grothe et al., 1999).

2.5 Poly(β-hydroxybutyric acid)

A gravimetric method similar to those employed previously (Marchessault et al., 1990; Ramsay et al., 1990; Grothe et al., 1999) was used. A sodium dodecyl sulfate (SDS) solution (1% w/v SDS, 10 ml, pH 10) was added to the biomass pellet obtained as described for cell dry mass measurements. The mixture was incubated on an orbital shaker (60 min, 200 rpm, 37 °C). The solids were recovered by centrifugation (4 min, 7,000 g) and washed with commercial sodium hypochlorite solution (Javex-5, Colgate-Palmolive Canada Inc., Toronto; 1 ml, 5.64% w/v sodium hypochlorite) that had been diluted to 20 ml. The pellet was centrifuged (4 min, 7,000 g), washed with deionized water (20 ml), and centrifuged again. The final pellet was dried (90 °C, 24 h) to constant weight in preweighed aluminum dishes (Grothe et al., 1999). The PHB yield coefficient relative to biomass (Y_{PBX}) was calculated as the mass of PHB obtained per unit cell dry weight. Measurements were in duplicate.

3 Results and discussion

3.1 Constant rate and exponentially increasing feed rate cultures

In batch culture, Medium 1018 (Table 1) is known to yield biomass with ~62% PHB content, but Medium 3 provides a higher total amount of PHB because it supports a substantially greater quantity of biomass than does Medium 1018 (Grothe et al., 1999). Similarly, Medium 3 significantly outperforms Medium 1 and Medium 2 (Table 1) in providing a greater final amount of biomass and PHB for otherwise identical conditions (Grothe et al., 1999). Medium 2 had been developed (Grothe et al., 1999) using a published elemental analysis of A. latus (Table 2) as the basis. Medium 2 is richer in nitrogen compared to the other media listed (Table 1) and it is quite low in phosphates. With respect to trace elements, Medium 2 is generally richer than the other media. Media 1 and 3 were used in fed-batch experiments.
Table 2. Composition of A. latus (percent of dry weight)

<table>
<thead>
<tr>
<th>Element</th>
<th>Percent</th>
<th>C:E ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>47.90</td>
<td>1.0</td>
</tr>
<tr>
<td>Oxygen</td>
<td>34.78</td>
<td>1.4</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>6.92</td>
<td>6.9</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>6.22</td>
<td>7.7</td>
</tr>
<tr>
<td>Sodium</td>
<td>1.25</td>
<td>38.3</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>1.045</td>
<td>45.8</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.225</td>
<td>212.9</td>
</tr>
<tr>
<td>Sulfur</td>
<td>0.175</td>
<td>273.7</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.163</td>
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</tr>
<tr>
<td>Iron</td>
<td>2.23</td>
<td>21.5</td>
</tr>
<tr>
<td>Calcium</td>
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<tr>
<td>Cobalt</td>
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<tr>
<td>Manganese</td>
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</tr>
<tr>
<td>Boron</td>
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<td>17741</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.0013</td>
<td>36846</td>
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<tr>
<td>Nickel</td>
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<td>36846</td>
</tr>
<tr>
<td>Copper</td>
<td>0.0005</td>
<td>95800</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>0.0001</td>
<td>479000</td>
</tr>
</tbody>
</table>

Source: Yamane et al. (1996)

The first experimental fed-batch culture was fed at a constant rate of 50 ml · h⁻¹ commencing after 33 h of batch operation. The feed was concentrated Medium 1 (Table 1) that contained 50% sucrose and 10 ml TES 1 per liter, so that the sucrose feed rate was 5 g · l⁻¹ · h⁻¹. The selection of the feed rate was based on an expected final biomass yield coefficient and nitrogen source, apparently contributed to the onset of the decline phase. Throughout, the nitrogen had declined to quite low levels, but more than 10 g · l⁻¹ sucrose remained (Fig. 1). At 68 h when feeding ceased, the cell concentration was ~27 g · l⁻¹ and PHB constituted 31% of the cell dry weight. The maximum specific growth rate was 0.105 h⁻¹. At 68 h the culture entered a decline phase although sufficient carbon and nitrogen were available. The constant feed rate strategy was unsatisfactory as the carbon- and nitrogen substrates accumulated in the broth (Fig. 1). The rapid pickup in growth upon commencement of feeding (Fig. 1) is apparently due to alleviation of nitrogen limitation: by 20 h the nitrogen had declined to quite low levels, but more than 10 g · l⁻¹ sucrose remained (Fig. 1). The sucrose concentration in the bioreactor after inoculation (5 l). The rapid increase in growth upon commencement of feeding (Fig. 1) was closely followed the empirical equation:

\[ t \text{-intercept) = 12.52 g \text{· l}^{-1} \cdot h^{-1} \]

where the best-fit values of the various parameters were: A (the y-intercept) = 12.52 g · l⁻¹; B (slope) = 16.53

The fermentation had to be stopped after 12 h using Medium 1. The fermentation had to be stopped after 12 h using Medium 1; the cells had 63% PHB by weight, or more than 4.4 g · l⁻¹ · h⁻¹ in terms of sucrose. The culture (Fig. 2) employed lower initial sucrose and nitrogen levels than the earlier described fermentation (Fig. 1). The sucrose concentration never exceeded ~15 g · l⁻¹. Ammonium sulfate was the nitrogen source in the batch phase. The initial C:N ratio was 28.3. Both the nitrogen source and the C:N ratio used had been established as optimal in earlier work (Grothe et al., 1999). A second fermentation (Fig. 2) utilized Medium 3 at a lower constant feed rate than earlier, in attempts to reduce possible substrate inhibition. Note that carbon and nitrogen associated inhibition has been reported with A. eutrophus (Belfares et al., 1995), a microorganism that is related to A. latus used here. The feed rate was 44 ml · h⁻¹, or 4.4 g · l⁻¹ · h⁻¹ in terms of sucrose. The culture (Fig. 2) employed lower initial sucrose and nitrogen levels than the earlier described fermentation (Fig. 1). The sucrose concentration never exceeded ~15 g · l⁻¹. Ammonium sulfate was the nitrogen source in the batch phase. The initial C:N ratio was 28.3. Both the nitrogen source and the C:N ratio used had been established as optimal in earlier work (Grothe et al., 1999). The cell concentration attained after 12 h was 28.3. Both the nitrogen source and the C:N ratio used had been established as optimal in earlier work (Grothe et al., 1999). A second fermentation (Fig. 2) utilized Medium 3 at a lower constant feed rate than earlier, in attempts to reduce possible substrate inhibition. Note that carbon and nitrogen associated inhibition has been reported with A. eutrophus (Belfares et al., 1995), a microorganism that is related to A. latus used here. The feed rate was 44 ml · h⁻¹, or 4.4 g · l⁻¹ · h⁻¹ in terms of sucrose. The culture (Fig. 2) employed lower initial sucrose and nitrogen levels than the earlier described fermentation (Fig. 1).
As opposed to the constant feed rates of Figs. 1 and 2, the fermentation shown in Fig. 3 employed an exponential feeding method using Medium 3. Thus, based on the data obtained in earlier fermentations, the following parameter values were assumed: total initial biomass content $X_0$ of 35 g equivalent to 7 g l$^{-1}$ biomass in the broth, a maximum specific growth rate ($\mu_{\text{max}}$) of 0.27 h$^{-1}$, and a biomass yield coefficient on sucrose ($Y_{X/S}$) of 0.37. The theoretical feeding profile was calculated using the equation:

$$F_s = \frac{X_0}{Y_{X/S}S_0} \cdot \mu e^{\mu t},$$

(2)

where $\mu$ is the specified growth rate, $t$ is the fermentation time, $X_0$ is the total amount of biomass in the fermenter at time zero, $F_s$ is the feed mass flow rate, and $S_0$ is the mass fraction of the substrate in the feed. The $S_0$ value was 0.5. Equation (2) assumes first order growth kinetics, i.e.:

$$X_t = X_0 e^{\mu t},$$

(3)

where $X_t$ is the total amount of biomass in the fermenter at time $t$. Equation (2) is derived from a mass balance on the substrate in the fermenter; thus, the substrate consumption rate is expressed as:

$$-\frac{dS}{dt} = F_s S_0 - \frac{\mu}{Y_{X/S}} X_t - \frac{q_p}{Y_{P/S}} X_t - m X_t,$$

(4)

where $S$ is the total substrate in the fermenter at time $t$, $F_s \cdot S_0$ is the substrate supply term, and the other terms on the right-hand-side of the equation represent, respectively, the substrate consumption due to microbial growth, product formation, and maintenance metabolism. Here, $q_p$ is the specific product formation rate, and $Y_{P/S}$ is the product yield based on substrate. Generally, under conditions of exponential growth, the maintenance coefficient $m$ is quite small and the maintenance term may be neglected. Furthermore, because PHB is an intracellular product, the third term on the right-hand-side of Eq. (4) may be lumped with the second term; hence, Eq. (4) becomes:

$$-\frac{dS}{dt} = F_s S_0 - \frac{\mu X_t}{Y_{X/S}}.$$

(5)

When the substrate feed rate exactly matches the consumption rate, $dS/dt = 0$; hence, from Eq. (5) follows:

$$F_s = \frac{\mu X_t}{Y_{X/S}S_0}.$$

(6)

Substitution of Eq. (3) into Eq. (6) yields the theoretical feed profile, Eq. (2).

Apparently because of a high assumed value of $\mu$ in Eq. (2), the calculated substrate feed rate exceeded the consumption rate and the sucrose concentration in the fermenter rose rapidly (Fig. 3); thus, exponential feeding was terminated at 9.5 h. After 21 h of culture, feeding was resumed, but at a constant rate of 4.4 g l$^{-1}$ h$^{-1}$ until 45 h.
Around 10 h, most of the nitrogen had been depleted (Fig. 3) and the culture may have been nitrogen limited; hence, a reduced growth rate between 7–20 h (Fig. 3). With continuing nitrogen feed in response to the pH control signal, the nitrogen level picked up beyond 20 h and this may have contributed to increased growth rate after that time (Fig. 3). The sucrose level was always above the limiting value beyond 10 h of culture. At 7 h, the calculated maximum specific growth rate was 0.254 h⁻¹, or lower than the value initially assumed for calculating the feed profile. After 48 h, the maximum biomass concentration was 16.3 g l⁻¹, corresponding to a product yield of ~50%. The maximum PHB productivity was estimated at 0.544 g l⁻¹ h⁻¹. The maximum sucrose consumption rate was 3.9 g l⁻¹ h⁻¹, or ~89% of the assumed value.

The pH regulation commenced at 7 h with the fed-batch phase (Fig. 3). Again, during pH regulation, the ammonium ion concentration increased exponentially with time according to the equation:

\[ C_{NH_4^+} = 11.2 \ e^{(t-14.8)/7.9}, \]

that confirmed \( C_{NH_4^+} \) as a direct feed-back indicator of the biomass growth. The product yield coefficient on biomass increased from 0.4 to 0.5 during culture (Fig. 3); however, the biomass yield on sucrose declined from a high value of 0.6 to 0.4. This meant that a greater percentage of sucrose was converted to PHB in the earlier half of the fermentation than in the later half. Toward the end, the PHB yield on sucrose was 16%.

3.2 pH-stat fed batch culture

One way of controlling pH in pH-stat fed-batch culture is to couple the feeding rate to the consumption of the pH control additive. For example, for an acid producing fermentation, the quantity of acid produced is a function of the growth rate and the amount of biomass (i.e., the rate of consumption of the carbon source); thus, the amount of alkali needed to neutralize the acid is proportional to the substrate consumption rate. In principle, feeding in response to demand of alkali for pH control should assure that the nutrients are fed in proportion to their rate of consumption; hence, the level of nutrients in the fermenter may be held nearly constant (Yamane et al., 1996). In the fed-batch phase, when the added ammonium is the sole nitrogen source, and at steady state, a biomass balance may be written as:

\[ C_C Y_{X/S} = C_N Y_{R/N} = C_T Y_{R/T}. \]

Here \( C_C, C_N, \) and \( C_T \) are the mass fractions of the carbon source, the nitrogen source, and trace elements in the respective feed solutions. The flow rates of the carbon, nitrogen, and the trace element feed streams are \( F_C, F_N, \) and \( F_T, \) respectively. The yield coefficient of the biomass (cells and intracellular PHB) on the carbon source is \( Y_{X/S}; \) and the yield coefficients of PHB-free biomass (or ‘residual’ biomass) on ammonium and trace elements are \( Y_{R/N} \) and \( Y_{R/T}, \) respectively. Note that the carbon source is consumed both in producing PHB and the PHB-free biomass, whereas PHB does not contain nitrogen and trace elements. From Eq. (8), the ratios of carbon-to-nitrogen and trace-elements-to-nitrogen feeds are readily shown to be:

\[ \frac{F_C}{F_N} = \frac{C_N}{C_C} \frac{Y_{R/N}}{Y_{X/S}}; \]

and

\[ \frac{F_T}{F_N} = \frac{C_N}{C_T} \frac{Y_{R/N}}{Y_{R/T}}, \]

respectively. Because

\[ \frac{Y_{X/S}}{1 - \delta} = \frac{Y_{R/S}}{1 - \delta}, \]

Equation (9) may be written as:

\[ \frac{F_C}{F_N} = \frac{C_N}{C_C} \frac{Y_{R/N}}{Y_{R/S}} (1 - \delta), \]

where \( \delta \) is the mass fraction of PHB in the cells.

A pH-stat method was used for the fed-batch phase of the fermentation profiled in Fig. 4. Approximately 1.5 l of 50% w/v sucrose solution and 160 ml of 20% w/v ammonia solution were fed from separate reservoirs held on balances (Mettler, PE11 or PM3000) and continuously stirred with a magnetic stirrer. The feeds were supplied by using a single peristaltic pump drive with two pump heads equipped with different diameter tubing to obtain the desired ratio of carbon-to-nitrogen feeds. The trace elements were fed with the sucrose medium that contained 10 ml l⁻¹ trace element solution. The pump was controlled in response to a signal from a pH controller.

Fig. 4. Time profile of A. latus fed-batch culture. A pH-stat fed-batch method was used with \( F_C/F_N = 10 \)
The fed-batch phase commenced as soon as the pH of batch culture dropped to below the pH set point of 6.5 (Fig. 4). Published values of \( Y_{X/S} = 0.37 \) and \( Y_{R/N} = 3.92 \) (Yamane et al., 1996) were used in Eq. (9) to estimate an expected value of \( F_{C/F_N} \) of 9.64; however, an experimental \( F_{C/F_N} \) value of 10 was used for the fermentation profiled in Fig. 4. Control of pH commenced at 17 h and feeding commenced at 27 h of culture when the pH had dropped to below the set point value of 6.5. As shown in Fig. 4, the concentration of sucrose did not remain constant although the pH was controlled effectively. Apparently, the estimated value of \( F_{C/F_N} \) was high because of discrepancies in the \( Y_{X/S} \) and \( Y_{R/N} \) values reported in the literature (Yamane et al., 1996) and those actually observed. Also, \( \delta \) and \( Y_{X/S} \) varied during the fermentation (Fig. 4) whereas they had been assumed constant in calculating the \( F_{C/F_N} \) ratio. The maximum final PHB content in the biomass was 53%. The maximum specific growth rate was estimated to be 0.149 h\(^{-1}\); the maximum rates of PHB synthesis and sucrose consumption were 0.782 and 3.57 g \( \cdot \) l\(^{-1} \) \( \cdot \) h\(^{-1}\), respectively.

The fermentation profiled in Fig. 5 also used a pH-stat feeding mode as explained previously, except that the value of the \( F_{C/F_N} \) ratio was reduced from 10 (Fig. 4) to 7 (Fig. 5). Consequently, a desired low sucrose concentration was maintained over a longer period (Fig. 5). The feeding and pH regulation commenced together at 24 h and ended at 40 h. For a short period near the end of the batch phase, insufficiency of nitrogen (Fig. 5, lower part) may have limited cell growth. The maximum specific growth rate was 0.154 h\(^{-1}\) and the final PHB content in biomass reached 52%. The PHB productivity \( q_P \) was 0.99 g \( \cdot \) l\(^{-1} \) \( \cdot \) h\(^{-1}\) for a sucrose consumption rate of 3.42 g \( \cdot \) l\(^{-1} \) \( \cdot \) h\(^{-1}\). The biomass yield on sucrose was about 40%. Both the constant rate fed cultures (Figs. 1, 2) accumulated sucrose, suggesting a greater than needed feed rate value.

### 3.3 Comparison of feeding methods

The five fermentations and the three feeding strategies used are compared in Table 3 in terms of several measures of process performance. In general, feeding methods that reduced or delayed accumulation of substrates led to higher productivity of PHB. Thus, at 0.97 g \( \cdot \) l\(^{-1} \) \( \cdot \) h\(^{-1}\) the mean PHB productivity of fermentations 2, 4 and 5 (Table 3) was more than twice a mean value of 0.43 g \( \cdot \) l\(^{-1} \) \( \cdot \) h\(^{-1}\) for fermentations 1 and 3. Also, from Table 3, while accumulation of substrate had a strong negative influence on PHB productivity, substrate accumulation had little effect on biomass productivity. The latter was within 22% of a mean value of 3.49 g \( \cdot \) l\(^{-1} \) \( \cdot \) h\(^{-1}\) for the five fermentations. Note from Table 3, that a low specific growth rate (e.g., in fermentation 1) is not a requirement for high accumulation of PHB, but a reduced substrate inhibition is. High specific growth rates combined with controlled feeding to reduce substrate accumulation enhance PHB productivity as in fermentations 2, 4, and 5. Consequently, a workable method of feeding, such that substrate supply does not limit growth yet there is no accumulation of substrate, is indicated for improving productivity and yield of PHB. Of the feeding methods

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Feed strategy</th>
<th>Feed strategy</th>
<th>Feed strategy</th>
<th>Feed strategy</th>
<th>Feed strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation no.</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Culture time (h)</td>
<td>68</td>
<td>12</td>
<td>51</td>
<td>48</td>
<td>46.5</td>
</tr>
<tr>
<td>Biomass (g ( \cdot ) l(^{-1}))</td>
<td>24.7</td>
<td>14.9</td>
<td>32.9</td>
<td>27.3</td>
<td>35.4</td>
</tr>
<tr>
<td>PHB (g ( \cdot ) l(^{-1}))</td>
<td>8.5</td>
<td>9.4</td>
<td>16.3</td>
<td>14.5</td>
<td>18.2</td>
</tr>
<tr>
<td>( \mu ) (h(^{-1}))</td>
<td>0.105</td>
<td>0.265</td>
<td>0.254</td>
<td>0.149</td>
<td>0.154</td>
</tr>
<tr>
<td>( \dot{q}_{P,\text{max}} ) (g ( \cdot ) l(^{-1} ) ( \cdot ) h(^{-1}))</td>
<td>0.31</td>
<td>1.15</td>
<td>0.54</td>
<td>0.78</td>
<td>0.99</td>
</tr>
<tr>
<td>( \dot{q}_{S,\text{max}} ) (g ( \cdot ) l(^{-1} ) ( \cdot ) h(^{-1}))</td>
<td>3.15</td>
<td>3.40</td>
<td>3.92</td>
<td>3.57</td>
<td>3.42</td>
</tr>
<tr>
<td>( Y_{P/X} )</td>
<td>0.31</td>
<td>0.63</td>
<td>0.49</td>
<td>0.53</td>
<td>0.51</td>
</tr>
<tr>
<td>( Y_{X/S} )</td>
<td>0.24</td>
<td>0.51</td>
<td>0.36</td>
<td>0.29</td>
<td>0.39</td>
</tr>
<tr>
<td>( Y_{P/S} )</td>
<td>0.15</td>
<td>0.32</td>
<td>0.16</td>
<td>0.15</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Fig. 5. Time profile of \textit{A. latus} fed-batch culture. A pH-stat fed-batch method was used with \( F_{C/F_N} = 7 \)
examined, pH-stat strategy was useful but not entirely satisfactory in controlling substrate accumulation. The methods used, invariably required a priori knowledge of parameters such as specific growth rates, yield coefficients, and the biomass concentration at the end of the batch phase. Because these parameters vary, the fermentations generally tended to accumulate the substrate at various rates.

One fed-batch culture method that potentially can feed to match the maximum substrate demand and does not cause substrate accumulation, is that of Oh et al. (1998). That method, based on on-line monitored substrate uptake rate, does not require assumptions or a priori data on specific growth rates, yield coefficients, or the biomass concentration. The method needs on-line measurements of dissolved oxygen, but does not require monitoring of the substrate concentration. In view of these advantages, the feeding method of Oh et al. (1998) is recommended as having the potential to enhance PHB productivity.

Compared to published data for the same strain in batch culture (Grothe et al., 1999), the highest observed maximum specific growth rate in fed-batch operation was 3.5 times more. Similarly, the maximum PHB production rate was nearly 8-fold greater than values observed in batch culture. Clearly, relatively controlled fed-batch cultivation of A. latus is superior to batch fermentation for producing PHB.

4 Conclusions
Fed-batch culture is superior to batch fermentation of A. latus in producing PHB. Among the various feeding options, those that reduce or delay accumulation of substrate substantially improve PHB productivity relative to cases when the substrate is allowed to build up in the broth. Thus, the maximum PHB production rate (up to 1.15 g · l⁻¹ · h⁻¹) was nearly 8-fold greater than values observed in batch operations. Under the best conditions, PHB constituted up to 63% of dry cell mass after 12 h of culture. The maximum PHB concentration attained was about 18 g · l⁻¹, but this value may be further enhanced by improved control of feeding. Feeding needs to be controlled so that microbial growth is not limited, yet the concentration of substrate in the broth remains low or increases slowly to a low upper limit so that the substrate inhibition of PHB synthesis is minimal.

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