Inhibitory Effect of Carbon Dioxide on the Fed-Batch Culture of *Ralstonia eutropha*: Evaluation by CO2 Pulse Injection and Autogenous CO2 Methods

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Abstract: In order to see the effect of CO2 inhibition resulting from the use of pure oxygen, we carried out a comparative fed-batch culture study of polyhydroxybutyric acid (PHB) production by *Ralstonia eutropha* using air and pure oxygen in 5-L, 30-L, and 300-L fermentors. The final PHB concentrations obtained with pure O2 were 138.7 g/L in the 5-L fermentor and 131.3 g/L in the 30-L fermentor, which increased 2.9 and 6.2 times, respectively, as compared to those obtained with air. In the 300-L fermentor, the fed-batch culture with air yielded only 8.4 g/L PHB. However, the maximal CO2 concentrations in the 5-L fermentor increased significantly from 4.1% (air) to 15.0% (pure O2), while it was only 1.6% in the 30-L fermentor with air, but reached 14.2% in the case of pure O2. We used two different experimental methods for evaluating CO2 inhibition: CO2 pulse injection and autogenous CO2 methods. A 10 or 22% (v/v) CO2 pulse with a duration of 3 or 6 h was introduced in a pure-oxygen culture of *R. eutropha* to investigate how CO2 affects the synthesis of biomass and PHB. CO2 inhibited the cell growth and PHB synthesis significantly. The inhibitory effect became stronger with the increase of the CO2 concentration and pulse duration. The new proposed autogenous CO2 method makes it possible to place microbial cells under different CO2 level environments by varying the gas flow rate. Introduction of O2 gas at a low flow rate of 0.42 vvm resulted in an increase of CO2 concentration to 30.2% in the exit gas. The final PHB of 97.2 g/L was obtained, which corresponded to 70% of the PHB production at 1.0 vvm O2 flow rate. This new method measures the inhibitory effect of CO2 produced autogenously by cells through the entire fermentation process and can avoid the overestimation of CO2 inhibition without introducing artificial CO2 into the fermentor. © 2003 Wiley Periodicals, Inc.

Keywords: CO2 inhibition; PHB synthesis; pure oxygen; *Ralstonia eutropha*; CO2 pulse; autogenous CO2

INTRODUCTION

There have been many reports regarding the inhibitory effects of carbon dioxide on the growth and metabolism of a wide range of microorganisms such as bacteria, yeast, and filamentous fungi (Dixon and Kell, 1989; Jones and Greenfield, 1982; McIntyre and McNeil, 1998). The previous studies focused on using CO2 to control the growth of pathogenic organisms or food spoilage organisms and protect the quality of food or water (Dixon and Kell, 1989; McIntyre and McNeil, 1998). Later, it was found that the fermentation progress could be affected by the CO2 produced by the microorganism itself (Dixon and Kell, 1989; McIntyre and McNeil, 1998). It has been found that the fermentation progress could be affected by the CO2 produced by the microorganism itself (Dixon and Kell, 1989; McIntyre and McNeil, 1998). Many studies on the mechanisms were summarized by Jones and Greenfield (1982). Among the several molecular species of CO2 in the liquid phase, the CO2 (aq) and HCO3− were generally responsible for the CO2 inhibition on microorganisms (Dixon and Kell, 1989; Jones and Greenfield, 1982). However, it is still difficult to find a single primary mechanism to explain the observed results (McIntyre and McNeil, 1998).

CO2 inhibition on cell growth and product formation in fermentation has been demonstrated. Two research groups
Microorganism and Medium

Ralstonia eutropha (formerly known as Alcaligenes eutrophus) NCIMB 11599 was used throughout this study. The seed culture medium composition and the initial medium composition for fed-batch culture are given in Table I. The trace element solution contains per liter: 10 g FeSO₄ (7H₂O), 2.25 g ZnSO₄ (7H₂O), 1.2 g CuSO₄ (5H₂O), 0.5 g MnSO₄ (5H₂O), 2 g CaCl₂ (2H₂O), 0.25 g Na₂B₄O₇ (7H₂O), 0.12 g (NH₄)₆Mo₇O₂₄, and 10 ml 35% HCl.

Culture Condition

Fed-batch cultures of R. eutropha were carried out in three different scale fermentors (5, 30, and 300 L). At 30°C the gas flow-rates were maintained at 1.0 vvm based on the initial culture volume and pH was maintained at 6.7. Gas flow-rates and pressures were monitored and independently controlled with a gas mixer and a mass flow meter. When pure oxygen was used as the gas phase, the dissolved oxygen concentration was maintained at higher than 25% of air saturation by increasing the agitation speed. When air was used as the gas phase, the dissolved oxygen concentration was maintained as high as possible by increasing the agitation speed up to its maximum value. The maximum agitation speeds of 5, 30, and 300 L fermentors were 900, 450, and 210 rpm, respectively.

In checking the effects of carbon dioxide on the process of PHB production, a 6-h pulse of CO₂ with 22% (v/v) in pure oxygen was introduced into fermentor at a defined cell growth stage (lag, exponential, stationary). A 3-h pulse of CO₂ with 22% (v/v), and another 3-h pulse of CO₂ with 10% (v/v), were employed to examine the effects of CO₂ concentrations and CO₂ pulse lasting time on the fermentation process of PHB production. The gas mixture of CO₂ and pure oxygen was prepared with a gas-mixer (New Brunswick Scientific, Edison, NJ).

In the absence of oxygen limitation, the CO₂ concentration in the exit gas from a fermentor would increase with the decrease of the gas flow rate and the microbial cells can be placed under different CO₂ concentration environments, then the effect of CO₂ produced autogenously by cells can be estimated by changing the gas flow rate. Based on this

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Seed culture (g/L)</th>
<th>Fermentation medium (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>MgSO₄(7H₂O)</td>
<td>0.2</td>
<td>1.2</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Citric acid</td>
<td>9</td>
<td>1.7</td>
</tr>
<tr>
<td>Trace element</td>
<td>Solution (mL/L)</td>
<td>1</td>
</tr>
</tbody>
</table>

Table I. Defined medium compositions.
idea, a fed-batch culture was carried out at an O2 flow rate of 0.42 vvm to examine the effect of CO2 produced by *R. eutropha*.

### Analysis Methods

Cell growth was monitored by measuring an optical density (OD) at 600 nm with a spectrophotometer (Beckman, Palo Alto, CA). Cell concentration was determined by measuring the dry cell weight (DCW) of a 2-ml culture broth. The broth sample was centrifuged, washed with distilled water, and dried in an oven at 60°C until no further decrease was shown in weight. PHB concentration was determined with a gas chromatograph (Varian 3300, San Fernando, CA) with benzoic acid as the internal standard (Braunegg et al., 1978; Shang et al., 2003). The residual cell weight (RCW) was defined as the cell concentration less PHB concentration. Carbon dioxide concentration in the influent and effluent gas was measured with a gas analyzer (Model LKM2000-03, LOKAS Automation Corp., Korea). Glucose concentration in the medium was automatically analyzed and controlled at 9 g/L with a glucose analyzer (Model 2730, Yellow Springs Instruments, Youngstown, OH).

### RESULTS AND DISCUSSION

#### High Cell Density Culture With Pure Oxygen and CO2 Production

A set of fed-batch cultures of *R. eutropha* was carried out with pure oxygen or air, as shown in Table II. When air was used, the DCW and PHB concentration obtained in the 5-L fermentor decreased by 54% and 66%, respectively, as compared with those obtained with pure oxygen. In the 30-L fermentor, the DCW and PHB concentration decreased by 73% and 84%, respectively. In the 300-L fermentor, the obtained DCW was just 23.4 g/L due to the oxygen limitation. The supply of pure oxygen can effectively eliminate the oxygen limitation and achieve a higher cell density.

High cell density culture (HCDC) technologies have attracted attention, as they may guarantee high product level (Babu et al., 2000; Riesenberg and Guthke, 1999). It is relatively easy to achieve a very high cell density in a small-scale fermentation for several important industrial microorganisms such as *Escherichia coli*, yeast, *Bacillus*, and *R. eutropha*. However, with the decrease in the cost of producing high-purity oxygen, it would become possible to achieve a high cell density in large-scale fermentors as well. But the CO2 concentration in effluent gas will also increase in high cell density cultures. As shown in Table II, the ratio of exit CO2 concentrations in pure oxygen and air was just 3.6 in the 5-L fermentor, but it increased to 8.9 in the 30-L fermentor. In fact, the pure oxygen flow rate would be kept as low as possible to fully utilize O2. This would result in a further increase in the concentration of CO2. A highly dissolved CO2 concentration is more likely to occur in a large-scale industrial fermentor than in a lab-scale fermentor due to its hydraulic pressure.

#### CO2 Concentrations in the Effluent Gas and in the Culture Broth

A linear relationship between the dissolved CO2 concentration in the culture broth and that in exit gas was found when the operation pressure is steady (McIntyre and McNeil, 1997c). The liquid phase CO2 concentration can be monitored with a dCO2 probe. Its performance may be affected by the presence of organic acids, but not significantly (Puhar et al., 1980; McIntyre and McNeil, 1997c). Considering that CO2 in the culture broth can exist in various forms, such as CO2(aq), HCO3−, CO32−, and H2CO3, depending on pH, and they may affect the metabolic activity of microbial cells differently, we chose to monitor the concentrations of CO2 in the influent and effluent gas with a gas analyzer. At the current experimental pH of 6.7 the most dominant forms of CO2 appear to be CO2(aq) and HCO3−.

#### CO2 Inhibition on the Biosynthesis and Cell Growth

**Carbon Dioxide Pulses at Different Cell Growth Phases**

A series of experiments were designed to estimate the effects of CO2 on the cell growth and PHB formation in fed-batch cultures of *R. eutropha*. A 6-h pulse of 22% (v/v) CO2 in pure oxygen was introduced into fermentor at a defined cell growth stage such as the lag phase, the exponential phase, or the stationary phase to determine which is the most sensitive stage to CO2 based on cell growth and PHB formation (Fig. 1a–c). As a reference, a fed-batch culture was carried out at an O2 flow rate of 0.42 vvm to examine the effect of CO2 produced by *R. eutropha*.  

<table>
<thead>
<tr>
<th>Fermentors</th>
<th>Gas phase</th>
<th>Culture time (h)</th>
<th>RCW (g/L)</th>
<th>DCW (g/L)</th>
<th>PHB conc. (g/L)</th>
<th>CO2 conc. (%) (Max.)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 L</td>
<td>Oxygen</td>
<td>45.0</td>
<td>69.5</td>
<td>208.2</td>
<td>138.7</td>
<td>15.0</td>
<td>O2-cylinder</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>45.5</td>
<td>49.8</td>
<td>96.4</td>
<td>46.6</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>30 L</td>
<td>Oxygen</td>
<td>47.0</td>
<td>54.6</td>
<td>185.9</td>
<td>131.3</td>
<td>14.2</td>
<td>O2 from PSA</td>
</tr>
<tr>
<td></td>
<td>Air</td>
<td>45.5</td>
<td>27.9</td>
<td>49.2</td>
<td>21.3</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>300 L</td>
<td>Air</td>
<td>49.0</td>
<td>15.0</td>
<td>23.4</td>
<td>8.4</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Time course of OD, DCW, RCW, PHB concentration, and exit CO2 levels in the fed-batch cultures with a 6-hour CO2 pulse (22%) at a defined cell growth stage. a: Stationary stage between 34.5 and 40.5 h. b: Early exponential stage between 11 and 17 h. c: Lag stage between 3 and 9 h. d: Standard culture without CO2 pulse. OD (△), DCW (●), RCW (▲), PHB conc. (■), and exit CO2 conc. (●).
culture of *R. eutropha* was done with no CO₂ pulse (Fig. 1d).

Figure 1a shows the time course of OD, DCW, RCW, PHB, and exit CO₂ concentrations of the fed-batch culture with a CO₂ pulse introduced in the stationary stage. During the 6-h CO₂ pulse, the OD and DCW increased only a little in the beginning. The CO₂ concentration in the exit gas was about 33% (v/v) during the CO₂ pulse and it maintained at about 9% (v/v) thereafter. The decrease of OD and DCW may be attributed to the damage of cells caused by the temporarily elevated CO₂ concentration in the solution (Dixon and Kell, 1989; Jones and Greenfield, 1982; McIntyre and McNeil, 1997a, 1998). After the CO₂ pulse, the cell metabolic activity did not recover in the following 4 h. As a result, the final DCW and PHB concentration amounted to 176 and 118 g/L, respectively, which are lower than the DCW (208 g/L) and PHB concentration (138.7 g/L) obtained in the standard culture.

Figure 1b shows the inhibitory effect of a CO₂ pulse at the exponential phase. Although the maximal CO₂ concentration in the exit gas was just about 23% (v/v), lower than the 33% (v/v) mentioned above (Fig. 1a), the cell growth and PHB accumulation were severely influenced. During the CO₂ pulse, the DCW and PHB concentration only increased from 6.5–26 g/L and 0.8–2 g/L, respectively, as compared with the DCW increase of 7–48 g/L and PHB change of 0.6–6 g/L in the standard culture. After the CO₂ pulse ceased, the DCW and PHB concentrations increased continuously for 20.5 h, but did not increase further. The RCW continued to increase to 69 g/L in 34 h, which was obtained generally in about 22–24 h without a CO₂ pulse. This means that the average cell growth rate decreased to about one-third of the standard.

The lowest DCW and PHB concentrations were obtained with a CO₂ pulse in the lag stage between 3–9 h (Fig. 1c). The final DCW and PHB concentration just reached 138 and 78.8 g/L, respectively. In this case, it took more than 30 h when the RCW reached its maximal value. After the CO₂ pulse ceased, the cell growth and PHB accumulation were still slow. It can be said that the lag stage, in which the productivity of PHB is the lowest (Table III), is the stage most sensitive to the CO₂ pulse with 22% (v/v) CO₂ in the influent gas stream. A similar result was found in the culture of *A. niger* (McIntyre and McNeil, 1997c).

### Table III. Summary of the experimental results in the fed-batch cultures with CO₂ pulses.

<table>
<thead>
<tr>
<th>CO₂ pulses</th>
<th>Cell growth stage</th>
<th>CO₂ conc. in input gas (%) (v/v)</th>
<th>Pulse duration (h)</th>
<th>Fermentation time (h)</th>
<th>Final DCW (g/L)</th>
<th>Final PHB conc. (g/L)</th>
<th>Final PHB content (wt%)</th>
<th>Productivity of PHB (g/h.L)</th>
<th>Max. CO₂ conc. in exponential phase (%) (v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary</td>
<td>22</td>
<td>6</td>
<td>45</td>
<td>175.4</td>
<td>118.1</td>
<td>57.3</td>
<td>68.9</td>
<td>2.62</td>
<td>14.3</td>
</tr>
<tr>
<td>Exponential</td>
<td>22</td>
<td>6</td>
<td>45</td>
<td>143.4</td>
<td>84.2</td>
<td>59.2</td>
<td>56.5</td>
<td>1.87</td>
<td>—</td>
</tr>
<tr>
<td>Lag</td>
<td>22</td>
<td>6</td>
<td>45</td>
<td>138.3</td>
<td>78.8</td>
<td>59.5</td>
<td>56.9</td>
<td>1.75</td>
<td>—</td>
</tr>
<tr>
<td>Exponential</td>
<td>22</td>
<td>3</td>
<td>45</td>
<td>175.0</td>
<td>117.0</td>
<td>58.0</td>
<td>66.9</td>
<td>2.60</td>
<td>—</td>
</tr>
<tr>
<td>Exponential</td>
<td>10</td>
<td>3</td>
<td>40</td>
<td>198.4</td>
<td>122.0</td>
<td>67.4</td>
<td>64.1</td>
<td>3.05</td>
<td>—</td>
</tr>
<tr>
<td>No CO₂ pulse (standard culture)</td>
<td>45</td>
<td>208.2</td>
<td>138.7</td>
<td>69.4</td>
<td>66.3</td>
<td>3.08</td>
<td>15.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Low gas flow rate (0.42 vvm)</td>
<td>45</td>
<td>169.1</td>
<td>97.2</td>
<td>71.9</td>
<td>57.3</td>
<td>2.16</td>
<td>30.2</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
Effect of CO₂ Produced Autogenously by Cells

General CO₂ production pattern during aerobic fermentation. Microbial cells can release or utilize CO₂. Both CO₂ productions in the ethanol fermentation by *Saccharomyces cerevisiae* or *Zymomonas mobilis* and CO₂ utilization for PHB synthesis by *R. eutropha* in the presence of H₂ are product-related (Veeramallu and Agrawal, 1986; Ishizaki et al., 2001). If it is respiration-related, one mole of CO₂ is produced per mole of organic carbon oxidized. Since *R. eutropha* is aerobic, CO₂ will come from metabolic oxidation of glucose.

CO₂ production per liter of the culture broth depends on cell concentration in terms of g/L (X) and its specific metabolic activity (q CO₂). Thus, volumetric CO₂ production Q CO₂ will be represented as:

\[ Q_{CO₂} = q_{CO₂} X \]

Since X and q CO₂ are low in a lag phase, Q CO₂ is low, too. In a logarithmic growth phase q CO₂ is high and X increases exponentially. At some point of this logarithmic phase oxygen supply from air is not sufficient and thus pure oxygen is needed. Q CO₂ will increase and reach a maximum at the end of this phase. As the fermentation enters the stationary phase, microbial product synthesis are induced, Q CO₂ remains constant or decreases slowly.

Figure 1d shows that the maximal CO₂ concentration in exit gas from a 5-L fermentor was about 15% (v/v) when the gas flow-rate was 1.0 vvm based on the initial liquid volume. In a 30-L fermentor, the maximal CO₂ concentration in exit gas was 14.2% (v/v) (as shown in Table II). At the gas flow rate of 0.42 vvm the maximal CO₂ concentration from a 5-L fermentor reached 30.2% (v/v). In a pure oxygen system, enough oxygen is always present in the liquid media and Monod constant or critical oxygen concentration for oxygen is less than 1 mg O₂/L. It is unlikely that the oxygen uptake is transport-limited rather than reaction-limited. In this case a higher CO₂ concentration will occur at a lower flow rate.

Autogenous CO₂ on the PHB synthesis and cell growth.

In a large-scale industrial fermentor it is likely that the CO₂ concentration would be higher than that in a small-scale fermentor because of increased hydrostatic pressure (Onken and Liefke, 1989). It is very important to choose a suitable method to simulate the conditions in a large industrial fermentor for the estimation of CO₂ effect on microorganisms. McIntyre and McNeil (1997c) reported that the previous experimental method of continuous CO₂-enriched gassing of batch cultures from time zero may lead to a systematic overestimation of CO₂ inhibitory effects on mycelia organisms and proposed a CO₂ pulse method. The results obtained in the CO₂ pulse method clearly show that two parameters, CO₂ concentration and pulse duration, play a key role in the estimation of CO₂ inhibitory effect. However, these two parameters are chosen artificially, which may lead to no conclusive reasoning on how the result of a pulse test is related to real experimental conditions. Another problem in the CO₂ pulse method is the sudden increase of the CO₂ concentration that could not occur in actual fermentation processes.

Here we propose a new method to place microbial cells under a higher CO₂ concentration just by lowering the gas flow rate, without the necessity of introducing CO₂ artificially. As shown in Figure 4, the maximal CO₂ concentration reached 30.2% as the gas flow rate was maintained at 0.42 vvm. The final DCW (168 g/L) and PHB concentration (97.2 g/L) decreased by 18.8% and 29.9%, respectively, as compared with the results obtained in the standard culture (Fig. 1d). Also, the cell growth rate decreased by 39% when the CO₂ concentration was at its maximal value (30.2%). These decreases in DCW, PHB concentration, and cell growth rate were attributed to the inhibitory effect of the CO₂ produced by cells themselves. During the lag phase less inhibition occurred because of the low CO₂ concentration, but during the exponential and stationary phases severe inhibition could have occurred.

During fermentation with a gas flow rate of 0.42 vvm, it was clear that the inhibitory effect of CO₂ was higher than that in the case of a 3-h 22% CO₂ pulse, but lower than that in the case of a 6-h 22% CO₂ pulse. The maximal CO₂ concentration lasted for 6 h and showed a pattern of cell growth and CO₂ inhibition. This technique is essentially a natural combination of pulse and continuous injection tech-
<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Influent gas phases</th>
<th>Experimental conditions</th>
<th>CO₂ conc. in influent gas (%) (v/v)</th>
<th>CO₂ conc. in effluent gas (%) (v/v)</th>
<th>Final cell density (g/L)</th>
<th>Effects of CO₂</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Penicillium chrysogenum</em></td>
<td>Air and CO₂</td>
<td>Fed-batch culture; continuous CO₂ gassing</td>
<td>12.6</td>
<td>14.5</td>
<td>41.5</td>
<td>DCW decreased 24.5%; Penicillin conc. Decreased 40.0%</td>
<td>Ho and Smith, 1986</td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em></td>
<td>Air and CO₂</td>
<td>Fed-batch culture; continuous CO₂ gassing</td>
<td>20</td>
<td>21.8</td>
<td>16.5</td>
<td>DCW decreased 70.0%; Penicillin conc. Decreased 90.0%</td>
<td>Ho and Smith, 1986</td>
</tr>
<tr>
<td><em>Zymomonas mobilis</em></td>
<td>N₂</td>
<td>Anaerobic batch fermentation (CO₂ removal)</td>
<td>0</td>
<td>2.30 mmol/h (Max.)</td>
<td>2.8</td>
<td>Increase of about 15% in the average specific growth rate and about 12% in the cell-mass yield.</td>
<td>Veeramallu and Agrawal, 1986</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Air and N₂/CO₂</td>
<td>Continuous culture; (keeping a constant CO₂ conc. in exit gas)</td>
<td>—</td>
<td>4.4 – 20%</td>
<td>8.2 – 11</td>
<td>25% decrease in DCW; 8% increase in ethanol conc.</td>
<td>Kuriyama et al., 1993</td>
</tr>
<tr>
<td><em>Aspergillus niger A60</em></td>
<td>Air and CO₂</td>
<td>Batch culture; continuous gassing CO₂</td>
<td>7.5</td>
<td>—</td>
<td>11.8</td>
<td>35% decrease in DCW; and 65.4% decrease in citrate conc.</td>
<td>McIntyre and McNeil, 1997c</td>
</tr>
<tr>
<td><em>Aspergillus niger A60</em></td>
<td>Air and CO₂</td>
<td>Batch culture; 2.5-h CO₂ pulse at the exponential phase</td>
<td>7.5</td>
<td>—</td>
<td>17.5</td>
<td>3.8% decrease in DCW; and 11.5% decrease in citrate conc.</td>
<td>McIntyre and McNeil, 1997c</td>
</tr>
<tr>
<td><em>Aspergillus niger A60</em></td>
<td>Air and CO₂</td>
<td>Batch culture; 24-h CO₂ pulse in the lag phase from the beginning</td>
<td>7.5</td>
<td>—</td>
<td>13.0</td>
<td>28.6% decrease in DCW; and 48.7% decrease in citrate conc.</td>
<td>McIntyre and McNeil, 1997c</td>
</tr>
<tr>
<td><em>Ralstonia eutropha</em></td>
<td>Pure oxygen and CO₂</td>
<td>Fed-batch culture; 6-h CO₂ pulse at the exponential phase</td>
<td>22</td>
<td>24.3 (Max)</td>
<td>143.4</td>
<td>31.1% decrease in DCW; and 39.3% decrease in PHB productivity.</td>
<td>This work</td>
</tr>
<tr>
<td><em>Ralstonia eutropha</em></td>
<td>Pure oxygen</td>
<td>Fed-batch culture at a low flow rate</td>
<td>0</td>
<td>30.2 (Max.)</td>
<td>169.1</td>
<td>18.7% decrease in DCW; and 29.9% decrease in PHB productivity.</td>
<td>This work</td>
</tr>
</tbody>
</table>
techniques of variable CO₂ concentrations, while pulse and continuous injections applied constant CO₂ concentrations. This method provides an effective way to estimate the inhibitory effect of CO₂ on microorganisms.

CONCLUSION

High cell density culture of *R. eutropha* can be obtained by supplying pure oxygen. As a consequence, more CO₂ would be produced with the increase of cell density. This study confirmed the inhibitory effect of CO₂ on cell growth and PHB formation in the culture of *R. eutropha*, even though it is one of autotrophic microbes and can use CO₂ as the main carbon source to produce PHB. The inhibitory effect of CO₂ will become stronger with the increase of CO₂ pulse duration and CO₂ concentration. The lag-phase is the most sensitive stage to CO₂.

The marked difference found in the cultures with different CO₂ pulse duration and CO₂ concentration indicates the necessity of a suitable experimental method for the study of CO₂ inhibition. We developed a new technique of CO₂ inhibition study on fermentation, “the natural or autogenous CO₂ method” by varying the pure oxygen supply rate to make it possible to place microbial cells under different process CO₂ concentration environments. This method will serve as the estimation tool for CO₂ effect in a real fermentation process.

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