Inhibitory Effect of Carbon Dioxide on the Fed-Batch Culture of *Ralstonia eutropha*: Evaluation by CO_2 Pulse Injection and Autogenous CO_2 Methods

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Abstract: In order to see the effect of CO_2 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 O_2 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 CO_2 concentrations in the 5-L fermentor increased significantly from 4.1% (air) to 15.0% (pure O_2), while it was only 1.6% in the 30-L fermentor with air, but reached 14.2% in the case of pure O_2. We used two different experimental methods for evaluating CO_2 inhibition: CO_2 pulse injection and autogenous CO_2 methods. A 10 or 22% (v/v) CO_2 pulse with a duration of 3 or 6 h was introduced in a pure-oxygen culture of *R. eutropha* to investigate how CO_2 affects the synthesis of biomass and PHB. CO_2 inhibited the cell growth and PHB synthesis significantly. The inhibitory effect became stronger with the increase of the CO_2 concentration and pulse duration. The new proposed autogenous CO_2 method makes it possible to place microbial cells under different CO_2 level environments by varying the gas flow rate. Introduction of O_2 gas at a low flow rate of 0.42 vvm resulted in an increase of CO_2 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 O_2 flow rate. This new method measures the inhibitory effect of CO_2 produced autogenously by cells through the entire fermentation process and can avoid the overestimation of CO_2 inhibition without introducing artificial CO_2 into the fermentor. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* **83**: 312–320, 2003.

Keywords: CO_2 inhibition; PHB synthesis; pure oxygen; *Ralstonia eutropha;* CO_2 pulse; autogenous CO_2

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 CO_2 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 CO_2 produced by the microorganism itself (Dixon and Kell, 1989; McIntyre and McNeil, 1998; Onken and Liefke, 1989). Many studies on the mechanisms were summarized by Jones and Greenfield (1982). Among the several molecular species of CO_2 in the liquid phase, the CO_2 (aq) and HCO_3^- were generally responsible for the CO_2 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).

CO_2 inhibition on cell growth and product formation in fermentation has been demonstrated. Two research groups

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have reported the effects of dissolved CO_2 in two important industrial strains, *Penicillium chrysogenum* (Edwards and Ho, 1988; Ho and Smith, 1986) and *Aspergillus niger* (McIntyre and McNeil, 1997a,b,c, 1998). Their morphologies, being of importance in the evaluation of cell growth and product formation, were apparently affected if the influent CO_2 concentration was higher than 5%. Other organisms such as *Escherichia coli* (Lacoursiere et al., 1986; Mori et al., 1983), *Clostridium acetobutylicum* (Mollah and Stuckey, 1992), *Pseudomonas sp*. (Gill and Tan, 1979; King and Nagel, 1975), *Saccharomyces cerevisiae* (Kuriyama et al., 1993), and *Zymomonas mobilis* (Veeramallu and Agrawal, 1986) were also sensitive to CO_2. The reported effects were dependent on the CO_2 concentration and the organism employed in their experiments. In order to avoid the overestimation of CO_2 effects, a pulse or pulses of CO_2-enriched gas, rather than the continuous CO_2-enriched gassing of batch cultures from time zero, was used in batch cultures (McIntyre and McNeil, 1997c). The estimated CO_2 effects would depend on the CO_2 concentration and pulse lasting time in a given experiment. Therefore, the results obtained from continuous or CO_2 pulse technique may not be applicable to real fermentation conditions where autogenous CO_2 evolution is quite different from the experimental conditions.

A number of studies on the production of polyhydroxyalkanoates (PHAs) have been carried out (Kim et al., 1994; Lee et al., 2000; Hori et al., 2002; Park et al., 2001; Rehm and Steinbuchel, 1999; Ryu et al., 1997; Shang et al., 2003; Van Wegen et al., 2001). *Ralstonia eutropha* is one of the major producers of PHAs since it can grow fast and accumulate higher amounts of PHAs than any other microorganisms. The highest productivity (3.75 g PHB/L.h) was obtained in the fed-batch culture with the aid of pure oxygen (Ryu et al., 1999). However, the use of oxygen is expensive and thus it becomes necessary to maximize the use of pure oxygen by reducing the oxygen flow rate. This would result in a CO_2 concentration in culture media much higher than with air supply only. This high CO_2 concentration may inhibit the metabolic activities of microbial cells (Ho and Smith, 1986; McIntyre and McNeil, 1997c).

In this study we investigated the effects of CO_2 on cell growth and PHB synthesis in the fed-batch culture of *R. eutropha*. We applied the CO_2 pulse technique to see how CO_2 concentration and pulse duration affect PHB synthesis and cell growth. Our new proposed approach is to vary the oxygen flow rate so that the autogenous evolution of CO_2 from the microbial cells may change the CO_2 concentration in the culture broth. In this case, the CO_2 concentration will increase with the decrease of gas flow rate. The results of this study will shed light on temporal and integral CO_2 inhibition patterns on the biosynthesis of PHB and the growth of *R. eutropha.* This can give us insight into how to suppress or promote metabolic activities of various other microorganisms.

MATERIALS AND METHODS

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 $FeSO_4$ ($7H_2O$), 2.25 g $ZnSO_4$ ($7H_2O$), 1.2 g $CuSO_4$ ($5H_2O$), 0.5 g $MnSO_4$ ($5H_2O$), 2 g $CaCl_2$ ($2H_2O$), 0.25 g $Na_2B_4O_7$ ($7H_2O$), 0.12 g $(NH_4)_6Mo_7O_{24}$, 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 CO_2 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 CO_2 with 22% (v/v), and another 3-h pulse of CO_2 with 10% (v/v), were employed to examine the effects of CO_2 concentrations and CO_2 pulse lasting time on the fermentation process of PHB production. The gas mixture of CO_2 and pure oxygen was prepared with a gas-mixer (New Brunswick Scientific, Edison, NJ).

In the absence of oxygen limitation, the CO_2 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 CO_2 concentration environments, then the effect of CO_2 produced autogenously by cells can be estimated by changing the gas flow rate. Based on this

Table I. Defined medium compositions.

Chemicals	Seed culture (g/L)	Fermentation medium (g/L)
Glucose	10	20
$(NH_4)_2SO_4$	1	4
$MgSO_4(7H_2O)$	0.2	1.2
KH_2PO_4	1.5	4.5
Citric acid	9	1.7
Trace element Solution (mL/L)	1	10

idea, a fed-batch culture was carried out at an O_2 flow rate of 0.42 vvm to examine the effect of CO_2 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 CO_2 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 CO_2 concentration in effluent gas will also increase in high cell density cultures. As shown in Table II, the ratio of exit CO_2 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 O_2. This would result in a further increase in the concentration of CO_2. A highly dissolved CO_2 concentration is more likely to occur in a large-scale industrial fermentor than in a lab-scale fermentor due to its hydraulic pressure.

CO_2 Concentrations in the Effluent Gas and in the Culture Broth

A linear relationship between the dissolved CO_2 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 CO_2 concentration can be monitored with a dCO_2 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 CO_2 in the culture broth can exist in various forms, such as $CO_2^{(aq)}$, HCO_3^-, CO_3^{2-}, and H_2CO_3, depending on pH, and they may affect the metabolic activity of microbial cells differently, we chose to monitor the concentrations of CO_2 in the influent and effluent gas with a gas analyzer. At the current experimental pH of 6.7 the most dominant forms of CO_2 appear to be $CO_2^{(aq)}$ and HCO_3^-.

CO_2 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 CO_2 on the cell growth and PHB formation in fed-batch cultures of *R. eutropha*. A 6-h pulse of 22% (v/v) CO_2 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 CO_2 based on cell growth and PHB formation (Fig. 1a–c). As a reference, a fed-batch

Table II. Summary of fed-batch cultures with oxygen and air.

Fermentors	Gas phase	Culture time (h)	RCW (g/L)	DCW (g/L)	PHB conc. (g/L)	CO_2 conc. (%) (Max.)	Notes
5 L	Oxygen	45.0	69.5	208.2	138.7	15.0	O_2-cylinder
	Air	45.5	49.8	96.4	46.6	4.1	
30 L	Oxygen	47.0	54.6	185.9	131.3	14.2	O_2 from PSA
	Air	45.5	27.9	49.2	21.3	1.6	
300 L	Air	49.0	15.0	23.4	8.4	—	

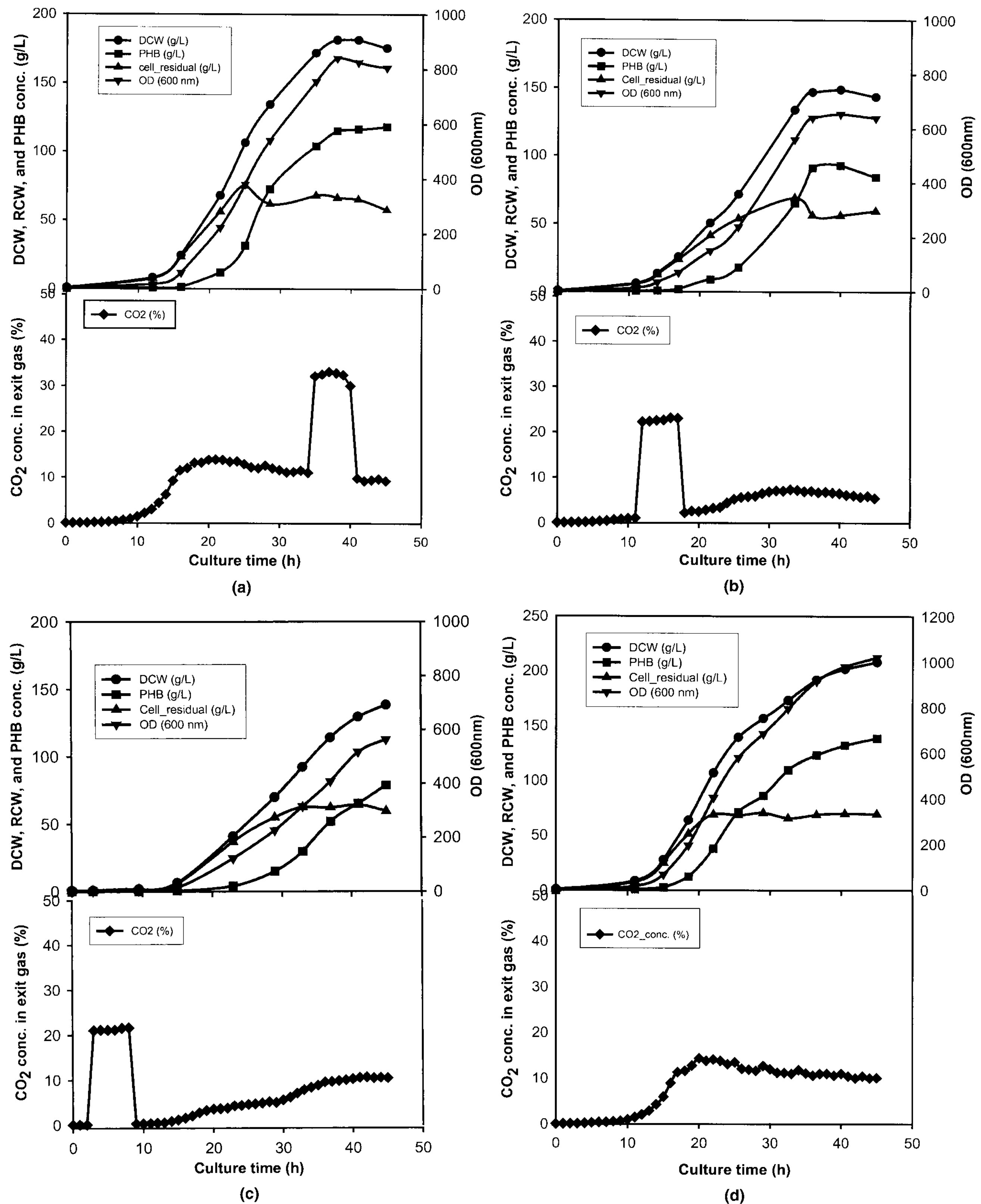

Figure 1. Time course of OD, DCW, RCW, PHB concentration, and exit CO_2 levels in the fed-batch cultures with a 6-hour CO_2 pulse (22%) at a defined cell growth stage. **a:** Stationary stage between 34.5 and 40.5 h. **b:** Early exponential stage between 11 and 17 h. **c:** Lag stage between 3 and 9 h. **d:** Standard culture without CO_2 pulse. OD (▼), DCW (●), RCW (▲), PHB conc. (■), and exit CO_2 conc. (♦).

culture of *R. eutropha* was done with no CO_2 pulse (Fig. 1d).

Figure 1a shows the time course of OD, DCW, RCW, PHB, and exit CO_2 concentrations of the fed-batch culture with a CO_2 pulse introduced in the stationary stage. During the 6-h CO_2 pulse, the OD and DCW increased only a little in the beginning. The CO_2 concentration in the exit gas was about 33% (v/v) during the CO_2 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 CO_2 concentration in the solution (Dixon and Kell, 1989; Jones and Greenfield, 1982; McIntyre and McNeil, 1997a, 1998). After the CO_2 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 CO_2 pulse at the exponential phase. Although the maximal CO_2 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 CO_2 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 CO_2 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 CO_2 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 CO_2 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 CO_2 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 CO_2 pulse with 22% (v/v) CO_2 in the influent gas stream. A similar result was found in the culture of *A. niger* (McIntyre and McNeil, 1997c).

Effects of CO_2 Pulse Duration and CO_2 Concentration

A 3-h CO_2 pulse was added into the fermentor at exponential phase to see the effect of CO_2 pulse lasting time in comparison with the 6-h CO_2 pulse (Fig. 2). The early exponential phase was chosen to introduce the CO_2 pulse since a large amount of CO_2 would be produced at this phase, not at the lag phase.

During the CO_2 pulse, the CO_2 concentration was about 23% (v/v), which is similar to the case of the 6-h CO_2 pulse. After 25 h, the CO_2 concentration was almost constant at 10% (v/v). The final DCW and PHB concentration reached 175 and 117 g/L, respectively; concentrations that are much higher than those obtained in the case of the 6-h CO_2 pulse (Fig. 1b). This shows that the pulse duration of CO_2 has a strong effect on cell growth and PHB accumulation. The longer the pulse duration, the stronger the inhibition of CO_2 on cell growth and production formation was found to be. In the culture of *A. niger*, the CO_2 inhibition on cell growth and critic acid synthesis also increased with the CO_2 pulse lasting time, as shown in Table IV.

Another 3-h pulse of 10% (v/v) CO_2 in pure oxygen was introduced at the early exponential phase to see the effect of CO_2 concentration on the fermentation process (Fig. 3). The CO_2 pulse lasted only for 3 h and the maximal CO_2 concentration in the exit gas was about 11% (v/v). Finally, it maintained at a constant value of 9% (v/v), and lasted about 20 h, which is very similar to the result obtained with the 3-h CO_2 pulse with 22% (v/v) CO_2 in pure oxygen (Fig. 2). The final DCW (190 g/L) and PHB concentration (122 g/L) were much higher than those obtained in the 3-h 22% (v/v) CO_2 pulse. From the results shown in Table IV, we can see the CO_2 inhibition in the cultures of *P. chrysogenum, Z. mobilis*, and *S. cerevisiae* also increased with the increase of CO_2 concentration. All these clearly showed that the higher the CO_2 concentration, the stronger the inhibitory effect of CO_2 on cell growth and production formation was.

The efficient way to prevent CO_2 inhibition is to lower the exit CO_2 concentration from a fermentor by either increasing the gas flow rate or supplying inert gases into the fermentor. In the former case a waste of pure oxygen is expected and in the latter case it will result in a low O_2 partial pressure.

Table III. Summary of the experimental results in the fed-batch cultures with CO_2 pulses.

CO_2 pulses									
Cell growth stage	CO_2 conc. in input gas (%) (v/v)	Pulse duration (h)	Fermentation time (h)	Final DCW (g/L)	Final PHB conc. (g/L)	Final RCW (g/L)	Final PHB content (wt%)	Productivity of PHB (g/h.L)	Max. CO_2 conc. in exponential phase (%) (v/v)
Stationary	22	6	45	175.4	118.1	57.3	68.9	2.62	14.3
Exponential	22	6	45	143.4	84.2	59.2	56.5	1.87	—
Lag	22	6	45	138.3	78.8	59.5	56.9	1.75	—
Exponential	22	3	45	175.0	117.0	58.0	66.9	2.60	—
Exponential	10	3	40	198.4	122.0	67.4	64.1	3.05	—
No CO_2 pulse (standard culture)			45	208.2	138.7	69.4	66.3	3.08	15.0
Low gas flow rate (0.42 vvm)			45	169.1	97.2	71.9	57.3	2.16	30.2

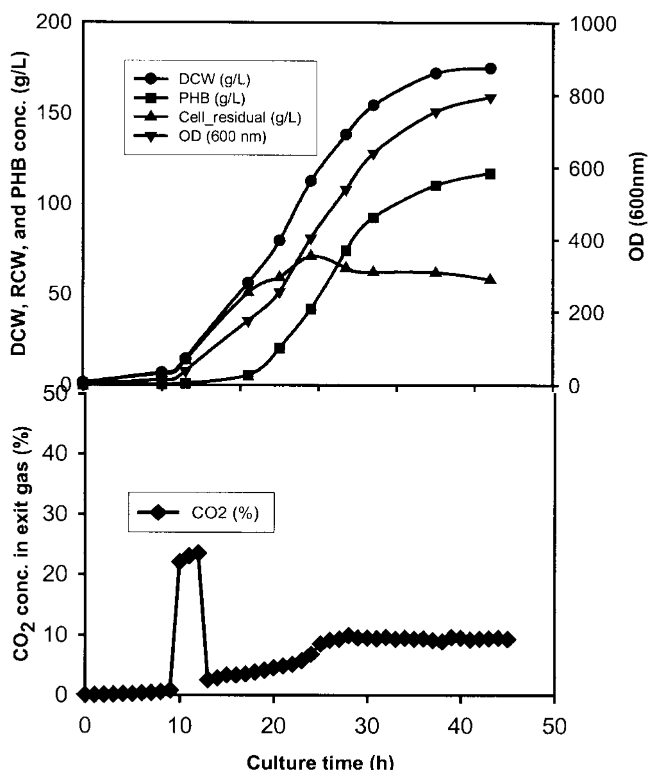

Figure 2. Time course of OD, DCW, RCW, PHB concentration, and exit CO_2 levels in the fed-batch culture with a 3-h CO_2 pulse (22%) in the early exponential stage between 10 and 13 h. OD (▼), DCW (●), RCW (▲), PHB conc. (■), and exit CO_2 conc. (♦).

Effect of CO_2 Produced Autogenously by Cells

General CO_2 production pattern during aerobic fermentation. Microbial cells can release or utilize CO_2. Both CO_2 productions in the ethanol fermentation by *Saccharomyces cerevisiae* or *Zymomonas mobilis* and CO_2 utilization for PHB synthesis by *R. eutropha* in the presence of H_2 are product-related (Veeramallu and Agrawal, 1986; Ishizaki et al., 2001). If it is respiration-related, one mole of CO_2 is produced per mole of organic carbon oxidized. Since *R. eutropha* is aerobic, CO_2 will come from metabolic oxidation of glucose.

CO_2 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 CO_2 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 CO_2 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 CO_2 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 CO_2 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 O_2/L. It is unlikely that the oxygen uptake is transport-limited rather than reaction-limited. In this case a higher CO_2 concentration will occur at a lower flow rate.

Autogenous CO_2 on the PHB synthesis and cell growth. In a large-scale industrial fermentor it is likely that the CO_2 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 CO_2 effect on microorganisms. McIntyre and McNeil (1997c) reported that the previous experimental method of continuous CO_2-enriched gassing of batch cultures from time zero may lead to a systematic overestimation of CO_2 inhibitory effects on mycelia organisms and proposed a CO_2 pulse method. The results obtained in the CO_2 pulse method clearly show that two parameters, CO_2 concentration and pulse duration, play a key role in the estimation of CO_2 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 CO_2 pulse method is the sudden increase of the CO_2 concentration that could not occur in actual fermentation processes.

Here we propose a new method to place microbial cells under a higher CO_2 concentration just by lowering the gas flow rate, without the necessity of introducing CO_2 artificially. As shown in Figure 4, the maximal CO_2 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 CO_2 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 CO_2 produced by cells themselves. During the lag phase less inhibition occurred because of the low CO_2 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 CO_2 was higher than that in the case of a 3-h 22% CO_2 pulse, but lower than that in the case of a 6-h 22% CO_2 pulse. The maximal CO_2 concentration lasted for 6 h and showed a pattern of cell growth and CO_2 inhibition. This technique is essentially a natural combination of pulse and continuous injection tech-

Table IV. Effects of CO_2 on the fermentation processes of several microorganisms.

Microorganisms	Influent gas phases	Experimental conditions	CO_2 conc. in fluent gas (%) (v/v)	CO_2 conc. in effluent gas (%) (v/v)	Final cell density (g/L)	Effects of CO_2	References
Penicillium chrysogenum	Air and CO_2	Fed-batch culture; continuous CO_2 gassing	12.6	14.5	41.5	DCW decreased 24.5%; Penicillin conc. Decreased 40.0%	Ho and Smith, 1986
Penicillium chrysogenum	Air and CO_2	Fed-batch culture; continuous CO_2 gassing	20	21.8	16.5	DCW decreased 70.0%; Penicillin conc. Decreased 90.0%	Ho and Smith, 1986
Zymomonas mobilis	N_2	Anaerobic batch fermentation (CO_2 removal)	0	230 mmol/h (Max.)	2.8	Increase of about 15% in the average specific growth rate and about 12% in the cell-mass yield.	Veeramallu and Agrawal, 1986
Saccharomyces cerevisiae	Air and N_2/CO_2	Continuous culture; (keeping a constant CO_2 conc. in exit gas)	—	4.4 ~ 20%	8.2 ~ 11	25% decrease in DCW; 8% increase in ethanol conc.	Kuriyama et al., 1993
Aspergillus niger A60	Air and CO_2	Batch culture; continuous gassing CO_2	7.5	—	11.8	35% decrease in DCW; and 65.4% decrease in citrate conc.	McIntyre and McNeil, 1997c
Aspergillus niger A60	Air and CO_2	Batch culture; 2.5-h CO_2 pulse at the exponential phase	7.5	—	17.5	3.8% decrease in DCW; and 11.5% decrease in citrate conc.	McIntyre and McNeil, 1997c
Aspergillus niger A60	Air and CO_2	Batch culture; 24-h CO_2 pulse in the lag phase from the beginning	7.5	—	13.0	28.6% decrease in DCW; and 48.7% decrease in citrate conc.	McIntyre and McNeil, 1997c
Ralstonia eutropha	Pure oxygen and CO_2	Fed-batch culture; 6-h CO_2 pulse at the exponential phase	22	24.3 (Max)	143.4	31.1% decrease in DCW; and 39.3% decrease in PHB productivity.	This work
Ralstonia eutropha	Pure oxygen	Fed-batch culture at a low flow rate	0	30.2 (Max.)	169.1	18.7% decrease in DCW; and 29.9% decrease in PHB productivity.	This work

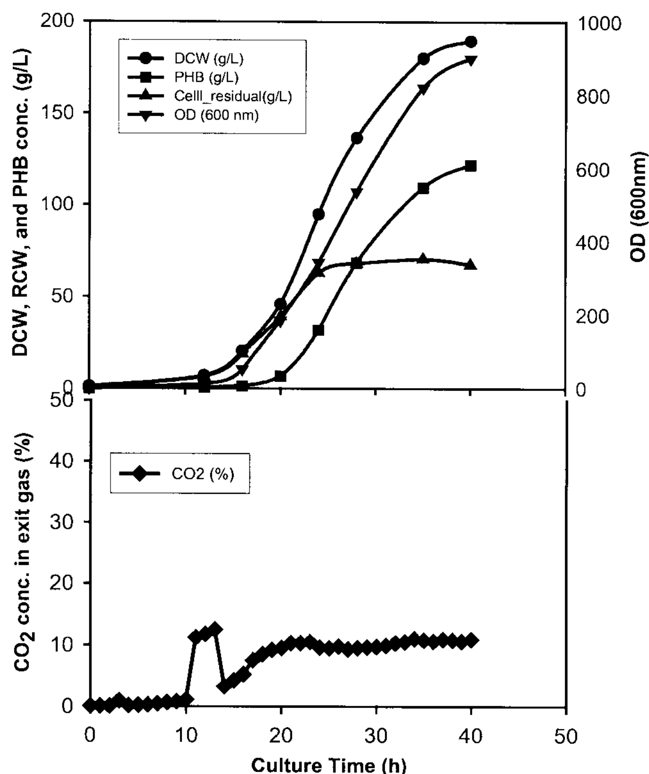

Figure 3. Time course of OD, DCW, RCW, PHB concentration and exit CO_2 levels in the fed-batch culture with a 3-h CO_2 pulse (10%) in the early exponential stage between 10.5 and 13.5 h. OD (▼), DCW (●), RCW (▲), PHB conc. (■), and exit CO_2 conc. (♦).

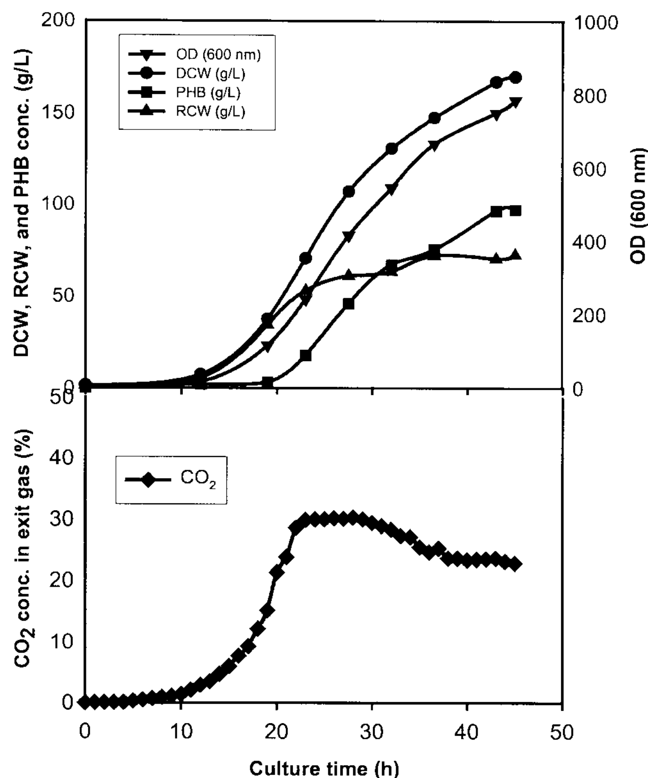

Figure 4. Time course of OD, DCW, RCW, PHB concentration, and exit CO_2 levels in the fed-batch culture at a gas flow rate of 0.42 vvm without a CO_2 pulse introduced. OD (▼), DCW (●), RCW (▲), PHB conc. (■), and exit CO_2 conc. (♦).

niques of variable CO_2 concentrations, while pulse and continuous injections applied constant CO_2 concentrations. This method provides an effective way to estimate the inhibitory effect of CO_2 on microorganisms.

CONCLUSION

High cell density culture of *R. eutropha* can be obtained by supplying pure oxygen. As a consequence, more CO_2 would be produced with the increase of cell density. This study confirmed the inhibitory effect of CO_2 on cell growth and PHB formation in the culture of *R. eutropha*, even though it is one of autotrophic microbes and can use CO_2 as the main carbon source to produce PHB. The inhibitory effect of CO_2 will become stronger with the increase of CO_2 pulse duration and CO_2 concentration. The lag-phase is the most sensitive stage to CO_2.

The marked difference found in the cultures with different CO_2 pulse duration and CO_2 concentration indicates the necessity of a suitable experimental method for the study of CO_2 inhibition. We developed a new technique of CO_2 inhibition study on fermentation, "the natural or autogenous CO_2 method" by varying the pure oxygen supply rate to make it possible to place microbial cells under different process CO_2 concentration environments. This method will serve as the estimation tool for CO_2 effect in a real fermentation process.

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