Effects of Elevated Dissolved CO₂ Levels on Batch and Continuous Cultures of *Aspergillus niger* A60: an Evaluation of Experimental Methods

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The effects of elevated levels of dissolved carbon dioxide (dCO₂), produced by gassing with CO₂-enriched gas mixtures, upon an industrial strain of *Aspergillus niger* (strain A60) producing citrate and gluconate were quantitatively assessed. Particular attention was paid to the reliability and accuracy of the steam-sterilizable dCO₂ probe, especially in the presence of high concentrations of potentially interfering acidic species. The response of the organism to elevated dCO₂ levels was assessed by using both batch and chemostat cultures, and the sensitivity of the organism in different growth phases (lag, exponential, and stationary) was examined. Chemostat cultures showed markedly less inhibition (in terms of biomass and organic acid synthesis) than did batch cultures. Studies in batch culture indicated that lag-phase cultures were especially sensitive to elevated dCO₂ levels. Overall, the results of this study indicate that previous experimental methods used to examine dCO₂ effects in submerged cultures (continuous CO₂-enriched gassing of batch cultures from time zero) have been inappropriate and have led to systematic overestimation of the inhibitory effects of dCO₂ on mycelial organisms.

The toxicity of carbon dioxide to a wide range of microorganisms has been demonstrated (8, 16). Initial studies of this process focused on the potential use of CO₂ as an agent to control the growth of pathogenic organisms (25) or food spoilage organisms (10, 22, 32). Later, it became apparent that organisms utilized in submerged liquid culture systems might also be subject to CO₂ inhibition. In these cases, CO₂ produced by the metabolism of the microorganism itself is the cause of the inhibition (24). CO₂ inhibition is likely to be most apparent in large fermentors (i.e., industrial-scale fermentors), in which increased hydrostatic pressure near the bases of the vessels results in increased dissolved CO₂ (dCO₂) concentrations (15).

Inhibitory effects of CO₂ in submerged bioprocesses have been well-documented, particularly in studies involving bacteria (11, 18, 23) and yeasts (5, 16, 17). Decreased growth (5, 8, 11, 16–18) and decreased product formation (8, 16, 23) are the most important effects observed.

Recently, there has been considerable interest in the effect of elevated dCO₂ levels in submerged liquid cultures of filamentous fungi, including *Penicillium chrysogenum* (13, 14, 26, 29, 30) and *Aspergillus niger* (20, 21). The general trend reported was that, as the CO₂ level in the influent gas phase was increased, growth and product formation decreased.

There may, however, be several fundamental problems with the techniques used in these studies. First, there is the question of which species of dCO₂ is responsible for the effects observed. It is only at pH levels below 4.0 that dCO₂ (i.e., CO₂*(aq)*) is the major species of CO₂ present (6). In the studies on *P. chrysogenum* (4, 13, 26, 29, 30) pH was controlled at 6.50, a value at which the inhibitory effects cannot be clearly ascribed to a single inhibitory species unambiguously.

An additional limitation of previous studies on *P. chrysogenum* was the fact there was no quantification of the morphological effect of CO₂. In the absence of such information, it is difficult to assess the precise effect of CO₂ in terms of morphological alterations. Given the profound impact of mycelial morphology on culture rheology and thus on mass momentum and heat transfer, this is unfortunate. Recent technological advances in tools for morphological analysis and in the application of these tools have emphasized the importance of such data in bioprocess monitoring (1, 4, 12, 31).

More recent studies on CO₂ effects (20, 21) on mycelial microorganisms in submerged culture have addressed some of the problems detailed above and have provided a greater understanding of the effects caused, particularly with reference to morphology.

With some of the difficulties in techniques and applications of technologies already dealt with, in the current study we attempted to advance this area of work further by addressing some of the remaining difficulties.

One piece of technology which has become available relatively recently is the steam-sterilizable dCO₂ probe (27), which is of great value in providing direct measurements of the concentration of dCO₂ (the inhibitory species) in a fermentation vessel. However, it has been reported that the performance of the dCO₂ probe may be affected by the presence of organic acids (27). As this could present a problem in investigations of the effects of elevated dCO₂ levels, a study of the probe characteristics is necessary. Another difficulty with previous studies on the effects of elevated CO₂ levels on fungi is the fact that these studies were usually carried out with batch cultures. Normally, the effects of elevated dCO₂ levels in late-exponential-phase industrial cultures have been simulated by gassing with CO₂-enriched air from the start of the laboratory scale batch process. This is almost certainly not an appropriate method, in view of the problems relating to the complex relationship between dCO₂ and gas phase CO₂ (see above) and also because lag-phase or early-exponential-phase cultures are exposed (inappropriately) to elevated dCO₂ levels. In a large-scale industrial process, high levels of CO₂ are present only
toward the ends of periods of rapid growth or when biomass levels are high (15), not from the start of a batch process.

In view of the difficulties with previous experimental methods, in the current investigation we attempted to determine the sensitivity of each of the growth phases of *A. niger* to CO₂ inhibition in batch fermentations. Our findings were then related to previous studies to determine their usefulness in simulating the response of actual cultures at the industrial scale. More realistic strategies for experimental design are also discussed below, particularly the use of chemostat continuous cultures, which has been seriously neglected in studies of CO₂ inhibition of microorganisms.

**MATERIALS AND METHODS**

dCO₂ probe. The response of a steam-sterilizable dCO₂ probe (Ingold) to 5 and 12% (vol/vol) CO₂ in air in the influent gas stream was monitored (Fig. 1). Calibration of the probe has been described previously (27). The starting point was a steady probe response to gassing with air (at a flow rate of 1 vol/vol min [vvm]), which returned to a steady response following sparging with a CO₂-air mixture. The probe output (in millibars) was recorded at 15-s intervals, and the CO₂-air mixture was replaced with air once a steady readout had been achieved with the gas mixture for 15 min.

The effect of citric acid on the probe response was monitored. The bioreactor was supplied with a gas mixture containing 5% (vol/vol) CO₂, and, when a steady readout had been achieved, citrate additions were begun. Aliquots of a stock solution of citrate were added to give final concentrations of 10, 20, 40, 60, 80, and 100 kg m⁻³ in the bioprocess broth. Successive additions of citrate were made following a steady probe response for 15 min. Probe response was again recorded at 15-s intervals. No pH change occurred upon citrate addition.

The conditions used for the probe experiments were the same as those used for the batch fermentations, as detailed below; the medium used was the medium used for the batch experiments, and the pH was 1.8.

Submerged liquid processes. (i) Microorganism. An industrial strain of *A. niger* (strain A60) was used in this study. Spore slants for inoculum purposes were prepared on potato dextrose agar (Unipath, Ltd.) in 0.02-dm³ universal bottles. Cultures for spore production were grown at 30°C for 7 days.

The medium used for inoculum development, batch processes, and the batch phase of continuous processes contained (per cubic meter) 140 kg of sucrose, 2.0 kg of KH₂PO₄, 2.0 kg of (NH₄)₂SO₄·7H₂O, 0.5 kg of MgSO₄·7H₂O, 0.1 × 10⁻² kg of ZnSO₄·7H₂O, 0.1 × 10⁻³ kg of (NH₄)₂SO₄·Fe₂(SO₄)₃·24H₂O, and 0.06 × 10⁻³ kg of CuSO₄·5H₂O. The pH was adjusted to 3.5.

The medium used for the continuous phase of chemostat studies was the same as the medium described above except that it contained (per cubic meter) 50 kg of sucrose, 1.0 kg of KH₂PO₄, 0.0 kg of (NH₄)₂SO₄, and 0.1 kg of MgSO₄·7H₂O. The pH was adjusted to 3.5.

The bioreactor inoculum was 0.8 dm³ of a 48-h-old shake flask culture grown at 30°C and 200 rpm.

(ii) Batch processes. The bioreactor used in this study was a Biostat ED ESIO bioreactor (B. Braun Biotech International) with a working volume of 8 dm³. Fermentations were performed at 30°C, and the stirrer speed was 300 rpm. The culture pH fell steadily to 1.80, reaching this value after between 43 and 74 h depending on the influent CO₂ concentration used.

The culture was aerated at a rate of 1 vvm. Gas flow rates and pressures were monitored and independently controlled by using a gas-mixing unit (model 881611; B. Braun Biotech International) and a thermal mass flow meter (B. Braun Biotech International). Processes were carried out with 2, 4, 7.5, 10, and 18% (vol/vol) CO₂ in air in the influent gas stream. The dynamic phase response of the culture to step changes in the influent CO₂ level was closely monitored.

In addition to a standard process with air as the input gas, processes were carried out with 2, 4, 7.5, 10, and 18% (vol/vol) CO₂ in air in the influent gas stream. The dynamic phase response of the culture to step changes in the influent CO₂ level was closely monitored.

(iii) Analyses. Dry weight was estimated by filtering 0.005 dm³ of culture broth through Whatman GF/C filter paper (diameter, 4.25 cm). Filter cakes were washed with 2 volumes of sterile distilled water, dried for 20 min in a microwave oven (650 W) on low power, and cooled before weighing.

The amount of sucrose in culture filtrates was determined by the method of Dubois et al. (9).

The citric acid concentration was determined by the method of Marier and Boulet (19).

The concentration of NH₄⁺ in culture filtrates was determined by using a kit (catalog no. 640) provided by Sigma, U.K. Ltd.

The concentration of carbon dioxide in the exit gas was monitored with a model ADC 7000 infrared gas analyzer (The Analytical Development Co.), and the concentration of oxygen in the fermentor offgas was monitored with a series 500 Servomex Paramagnetic oxygen analyzer (Sybron Taylor).
(iv) Morphological analysis. Morphological measurements were made by using a PC-based system and image analysis software (Aequitas Image Analysis; Dynamic Data Links). Images were captured with a monochrome video camera (model XC-77CE; DDC video camera module; Sony) connected to a microscope (model CH; Olympus) with a total magnification of $\times 400$. Measurements were made manually by using frozen images and a mouse to skeletonize individual hyphal trees (“organisms”) and to measure hyphal dimensions.

By using individual mycelial particles (hyphal trees), the mean main hyphal length, the mean branch length, and the mean hyphal growth unit were determined for each sample. The mean values were estimated from measurements of 50 mycelial particles for each fermentation sample.

**RESULTS**

The response of the dCO$_2$ probe was monitored when the influent gas contained a defined concentration of CO$_2$ in air. Figures 1a and b show the responses to 6 and 12% (vol/vol) CO$_2$ in air, respectively. In both cases the CO$_2$-containing gas mixture was introduced into the fermentor once a steady readout with air had been achieved, and the CO$_2$ mixture was replaced with air once a steady response had been achieved for 15 min.

Figures 1a and b show that after an initial lag the probe level reached 80% of its steady-state value within 5 min of being exposed to elevated CO$_2$ levels.

The effect of citrate on the probe response was also monitored (Fig. 1c) when the fermentor was sparged with a gas mixture containing 6% (vol/vol) CO$_2$ in air. Prior to the addition of citrate, a steady readout with 6% CO$_2$ had been achieved for 15 min. With additions up to the 40-kg m$^{-3}$ level, minor fluctuations in the probe response were observed, ($\pm$ 6% of the steady-state value). From around 100 min after citrate addition, however, the response was approximately steady, with only minor fluctuations in the recorded dCO$_2$ values.

A series of batch fermentations was carried out with a pulse of elevated CO$_2$ introduced into the fermentor at defined stages in the fermentation process (Fig. 2 through 4). In all pulse experiments CO$_2$ was introduced into the fermentor in the influent gas stream at a level of 7.5% (vol/vol) in air.

This set of experiments was designed to determine which stage of the fermentation was most sensitive to elevated levels
of dCO₂. Levels of dCO₂ equivalent to those produced by gassing with a gas mixture containing a CO₂ concentration of 7.5% may be expected in large-scale fermentors containing rapidly growing cultures or high levels of biomass (16). For this reason, pulses of 7.5% CO₂ lasting 2.5 h were introduced into batch cultures of *A. niger* in the early exponential phase (Fig. 2) and the stationary phase (Fig. 3). A third batch process experiment was carried out with *A. niger* which was exposed to an influent gas mixture containing 7.5% CO₂ for the first 24 h of cultivation to examine the effect of moderately elevated CO₂ levels on subsequent culture behavior. The time course for this process is shown in Fig. 4.

**DISCUSSION**

As can be seen from Fig. 1, the dCO₂ probe was shown to be a reliable means of measuring dCO₂ in fermentation broth. The probe response to changes in influent CO₂ level was rapid, with a good correlation between influent CO₂ level and the level of CO₂ dissolved in the liquid phase of the fermentor. Recently, there has been much discussion concerning the concentrations of carbon dioxide in a culture medium containing an active microorganism (2, 15, 28, 33). It is usually assumed that the concentration of CO₂ in the liquid phase in a bioreactor is in equilibrium with the concentration measured in the exit gas phase, that is, that Henry's law is applicable (2, 3). This assumption underpins many previous studies on effects of elevated CO₂ levels in submerged bioprocesses. However, it has been reported that under normal fermentation conditions, the level of CO₂ dissolved in the culture broth may be significantly higher than the equilibrium concentration, particularly when culture viscosity is high (7). The use of a dCO₂ probe and exit gas analysis in the present study, however, made it possible to measure the level of CO₂ dissolved in the liquid phase and the CO₂ concentration in the gas phase.

Results from this study show good correlation between gas and liquid phase CO₂ concentrations, with a linear relationship between the measured values for dCO₂ levels and exit CO₂ levels under both test conditions used (Fig. 1a and b) and also in the elevated CO₂ processes discussed in previous studies (20, 21).

Similarly, the results presented here (Fig. 1c) do not confirm...
previously stated reservations regarding the probe response being affected by the presence of organic acids (27). As Fig. 1c shows, minor fluctuations in probe response were observed when citrate was introduced into the fermentor at levels of 10 to 40 kg m$^{-3}$. However, the response returned to the steady-state value within 10 min. In an organic acid-producing fermentation system, such as a system in which citric acid is produced by *A. niger*, the rate at which citrate appears in the broth is slower and more gradual than the rate observed in the test. We concluded, therefore, that the probe was a reliable means of determining dCO$_2$ levels in cultures, even in the presence of citrate.

Once the reliability of the dCO$_2$ probe had been established, bioprocess experiments were performed with elevated CO$_2$ levels in the influent gas stream. The effects of exposure to a pulse of CO$_2$ on the growth and metabolism of *A. niger* A60 are shown in Fig. 2 through 4. By comparison with a standard batch process for this organism (20), no significant effect was observed on the culture exposed to 7.5% CO$_2$ for 2.5 h in both the early exponential phase and the stationary phase, and a significantly greater inhibitory effect was observed in the process exposed to 7.5% CO$_2$ for the first 24 h (the lag phase). However, in none of the pulse fermentations was the inhibitory effect as marked as the inhibitory effect in the process continuously exposed to 7.5% CO$_2$ (Table 1).

From the results presented here, it appears that a culture was most vulnerable to CO$_2$ inhibition in the lag phase. When a culture is inoculated into a fermentor, it is subject to many pressures due to the differences between the shake flask environment and the bioreactor environment, such as differences in pH and substrate concentration. When elevated dCO$_2$ levels in the fermentation fluid are also considered, it is likely that there are marked inhibitory effects on the culture. In the process exposed to elevated CO$_2$ levels during the lag phase (up to 24 h) (Fig. 4), a certain amount of recovery appears to have occurred once the stress of the early elevated CO$_2$ level was
abolically produced CO2 causes increases in dCO2 levels much
degrees of inhibition are likely to be of a magnitude greater
an unrealistic simulation method. In such studies, observed
Pulse 3 (first 24 h of lag phase)
industrial-scale cultures, it is unlikely that a culture growing on
entering the fermentor in the first 24 h of the process.
(i.e., the lag phase). A disproportionate level of inhibition is
likely to be present during the most sensitive phase of growth
process) with a set level of CO2 in the influent gas stream (4,
were continuously gassed (from time zero to the end of the
carbon dioxide levels in submerged liquid bioprocesses. Most
employed in previous studies to determine the effects of elevated
concentrations at the early stage of a process. It is only when
biomass levels or growth rates are high that levels of metabol-
and product levels influencing the observed effects. The much
reduced level of CO2 inhibition in chemostat cultures com-
pared with the level of CO2 inhibition noted in batch cultures
continuously gassed with elevated CO2 levels may be due to
the impact of CO2 inhibition in the early batch phase (lag and
early exponential phases), whereas, as Fig. 2 shows, exponential
growing cultures may be less sensitive to CO2 inhibition.

Conclusions. This study confirms the value of the dCO2
probe as a direct means of measuring the concentration of
dCO2 in submerged fungal cultures and indicates that probe
response is not interfered with by the presence of significant
concentrations of organic acids.
The results of this study call into question the relevance of
the most common previous experimental methods used to sim-
ulate elevated dCO2 levels. This is especially so because of the
sensitivity of the lag-phase culture to dCO2 inhibition.
Combined with the much lower levels of inhibition noted in
chemostat cultures, our findings indicate that inadequate or
inappropriate experimental methods have led to a systematic
overestimation of the inhibitory effects of elevated dCO2 levels
on microbial cultures in previous studies.

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TABLE 1. Reduction (relative to the control process value) in maximum biomass concentration (batch processes) or steady-state biomass concentration when the influent gas stream contained an elevated level of CO2.

<table>
<thead>
<tr>
<th>Conc of CO2 (% vol/vol) in influent gas stream</th>
<th>% Reduction in biomass concn relative to control process</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Batch processes</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>39</td>
</tr>
<tr>
<td>5</td>
<td>51</td>
</tr>
<tr>
<td>7.5</td>
<td>69</td>
</tr>
<tr>
<td>10</td>
<td>84</td>
</tr>
<tr>
<td>15</td>
<td>97</td>
</tr>
<tr>
<td>18</td>
<td>100</td>
</tr>
</tbody>
</table>

* Air was the sparge gas in the control.

TABLE 2. Maximum biomass concentration and maximum citrate concentration obtained when air (standard) or 7.5% CO2 was the sparge gas for the entire process or when the process was exposed to a pulse of CO2.

<table>
<thead>
<tr>
<th>Fermentation process</th>
<th>Maximum amt of biomass (kg m&lt;sup&gt;-3&lt;/sup&gt;)</th>
<th>Maximum citrate concn (kg m&lt;sup&gt;-3&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>18.2</td>
<td>78</td>
</tr>
<tr>
<td>7.5% CO2 continuous gassing</td>
<td>11.8</td>
<td>27</td>
</tr>
<tr>
<td>Pulse 1 (exponential phase)</td>
<td>17.5</td>
<td>69</td>
</tr>
<tr>
<td>Pulse 2 (exponential and stationary phases)</td>
<td>16.3</td>
<td>61</td>
</tr>
<tr>
<td>Pulse 3 (first 24 h of lag phase)</td>
<td>13.0</td>
<td>40</td>
</tr>
</tbody>
</table>

* See Fig. 2.
* See Fig. 3.
* See Fig. 4.


