Effect of elevated dissolved carbon dioxide concentrations on growth of *Corynebacterium glutamicum* on d-glucose and L-lactate

Carsten Bäumchen, Arnd Knoll, Bernward Husemann, Juri Seletzky, Bernd Maier, Carsten Dietrich, Ghassem Amoabediny, Jochen Büchs

Biochemical Engineering, RWTH Aachen University, D-52074 Aachen, Germany

Received 6 July 2006; received in revised form 20 December 2006; accepted 2 January 2007

Abstract

The effect of increased dissolved carbon dioxide concentrations on growth of *Corynebacterium glutamicum* was studied with continuous turbidostatic cultures. The carbon sources were either L-lactate or D-glucose. To increase the dissolved carbon dioxide concentration the carbon dioxide partial pressure of the inlet gas stream $p_{CO_2,IN}$ was increased stepwise from 0.0003 bar (air) up to 0.79 bar, while the oxygen partial pressure of the inlet gas stream was kept constant at 0.21 bar. For each resulting carbon dioxide partial pressure $p_{CO_2}$ the maximum specific growth rate $\mu_{max}$ was determined from the feed rate resulting from the turbidostatic control. On D-glucose and $p_{CO_2}$ up to 0.26 bar, $\mu_{max}$ was mostly constant around 0.58 h$^{-1}$. Higher $p_{CO_2}$ led to a slight decrease of $\mu_{max}$. On L-lactate $\mu_{max}$ increased gradually with increasing carbon dioxide partial pressures from 0.37 h$^{-1}$ under aeration with air to a maximum value of 0.47 h$^{-1}$ at a $p_{CO_2}$ of 0.26 bar. At very high $p_{CO_2}$ (0.81 bar) $\mu_{max}$ decreased down to 0.35 h$^{-1}$ independent of the carbon source.

Keywords: *Corynebacterium glutamicum*; Dissolved carbon dioxide; Carbon dioxide sensitivity; Carbon dioxide inhibition; Turbidostat culture; Pressure fermentation; High cell density cultivation (carboxylases; anaplerotic reactions)

1. Introduction

Due to the metabolic activity of growing cells in fermentations, the culture medium gets enriched with dissolved carbon dioxide (CO$_2$). Especially in industrial scale fermenters with high hydrostatic pressures, in high cell density fermentations or in high pressure fermentation processes inhibiting carbon...
Nomenclature

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>(c_{\text{CO}_2}^{\text{dissolved}})</td>
<td>dissolved carbon dioxide concentration (kmol m(^{-3}))</td>
</tr>
<tr>
<td>CTR</td>
<td>carbon dioxide transfer rate (kmol m(^{-3}) h(^{-1}))</td>
</tr>
<tr>
<td>(D)</td>
<td>dilution rate (h(^{-1}))</td>
</tr>
<tr>
<td>(G)</td>
<td>acceleration of gravity (m s(^{-2}))</td>
</tr>
<tr>
<td>(K_S)</td>
<td>affinity coefficient of the limiting substrate (kg m(^{-3}))</td>
</tr>
<tr>
<td>(L_{\text{CO}_2})</td>
<td>solubility of carbon dioxide (kmol m(^{-3}) bar(^{-1}))</td>
</tr>
<tr>
<td>(p_{\text{CO}_2})</td>
<td>CO(_2) partial pressure of the gas–liquid interface (bar)</td>
</tr>
<tr>
<td>(p_{\text{CO}_2,\text{IN}})</td>
<td>CO(_2) partial pressure of the inlet gas stream (bar)</td>
</tr>
<tr>
<td>(p_R)</td>
<td>system pressure of the reactor (bar)</td>
</tr>
<tr>
<td>(T)</td>
<td>temperature (°C)</td>
</tr>
<tr>
<td>(V_F)</td>
<td>feed consumption (L)</td>
</tr>
<tr>
<td>(y_i)</td>
<td>mole fraction of compound (i) (kmol kmol(^{-1}))</td>
</tr>
</tbody>
</table>

Greek letters

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\mu)</td>
<td>specific growth rate (h(^{-1}))</td>
</tr>
<tr>
<td>(\mu_{\text{max}})</td>
<td>maximum specific growth rate (h(^{-1}))</td>
</tr>
</tbody>
</table>

dioxide partial pressures may occur (Mostafa and Gu, 2003). For high pressure fermentations up to 11 bar the partial pressure of carbon dioxide can reach values of 0.8 bar or even higher (Knoll et al., 2005). These carbon dioxide partial pressures might have inhibitory effects on the growth of microorganisms (Jones and Greenfield, 1982; Dixon and Kell, 1989). In the last decade, non-thermal inactivation of microorganisms with high carbon dioxide partial pressures was a frequent topic in food-biotechnology (Spilimbergo and Bertucco, 2003). Such effects are not desired for fermentations processes and have to be avoided.

Thus, an appropriate process design is only possible if the response of the tested microorganism to different carbon dioxide partial pressures is known. This paper describes a method to determine the response of the maximum specific growth rate \(\mu_{\text{max}}\) at different carbon dioxide partial pressures using continuous turbidostatic cultures. The physiological effects of carbon dioxide on the metabolism of cells are discussed but were not investigated within this study.

* Corynebacterium glutamicum* is used as model organism to demonstrate the applicability of the method. *C. glutamicum* belongs to the large group of Gram-positive bacteria and is used predominantly for amino acid production such as L-lysine. The carbon flux through the central metabolic pathways and its importance for growth rate and product formation has been widely investigated over the last 20 years (Sahm et al., 2000). Within the metabolism the anaplerotic reactions are very important for replenishing the tricarboxylic acid cycle’s pool of metabolites. *C. glutamicum* has a highly sophisticated and regulated network of carboxylating and decarboxylating pathways to provide metabolites and cofactors. This interaction between anabolism and catabolism is a key parameter for growth and also product formation. The influence of CO\(_2\) on growth of *C. glutamicum* has never been investigated systematically. A method for the determination of the response of a microorganism to different carbon dioxide partial pressures would be a helpful tool for the investigation of the impacts on anaplerotic reactions. Therefore, the influence of carbon dioxide on growth has to be investigated independent of other factors and under steady-state conditions.

2. Theory

\(\text{D}-\text{glucose}\) is being taken up by *C. glutamicum* via phosphotransferase system (PTS) and is oxidized to pyruvate via both of the classic central metabolic pathways: glycolysis and pentose phosphate pathway (Dominguez et al., 1997). In contrast, \(\text{L}-\text{lactate}\) is utilized by \(\text{L}-\text{lactate}\) specific, membrane associated \(\text{L}-\text{lactate}\) dehydrogenase which is essential for growth of *C. glutamicum* on \(\text{L}-\text{lactate}\) as sole carbon source (Stansen et al., 2005). The catalytic activity of the \(\text{L}-\text{lactate}\) dehydrogenase is the oxidation of \(\text{L}-\text{lactate}\) to pyruvate. In the past metabolic flux analyses have shown that during utilization of \(\text{D}-\text{glucose}\) only low tricarboxylic acid cyle (TCA) activities were detectable (ca. 25% relative to acetate grown cells). In contrast, growth on the fermentation product acetate as sole carbon source showed high *in vivo* TCA cycle activities and down regulation of glycolytic genes and genes of the pentose phosphate shunt (Gerstmeir et al., 2003).
The higher TCA cycle activity in acetate grown cells partly compensates for the lower amount of energy which is provided per mol acetate.

But independent of the carbon source the TCA cycle is one of the main central pathways in C. glutamicum and other aerobic bacteria. The TCA cycle is responsible for the complete oxidation of acetyl-CoA derived from different substrates and for the provision of precursors for amino acid biosynthesis. During growth the TCA cycle has to be replenished continuously in order to maintain the acceptor molecule oxalacetate at a sufficient level and thus to keep the cycle running. For this purpose C. glutamicum possesses the anaplerotic reactions. Around the pyruvate node two important anaplerotic enzymes were identified: PEP carboxylase and pyruvate carboxylase which is the major carboxylating enzyme in C. glutamicum (Peters-Wendisch et al., 1997). In this work we studied the growth behaviour of C. glutamicum on the PTS sugar D-glucose and the fermentation product L-lactate under increasing carbon dioxide concentrations.

The influence of carbon dioxide on growth was determined by means of a continuous turbidostatic culture system. In a turbidostat biomass concentration is kept constant by a control loop, where biomass concentration or another analogous control variable such as optical density (Bryson, 1952), oxygen transfer rate, dielectric permittivity of the culture broth (Markx et al., 1991), pH value (Adamberg et al., 2003), dissolved oxygen concentration (Konstantinov et al., 1990), carbon dioxide output (Watson, 1972) or product concentration (Mühlemann and Bungay, 1993) is controlled by the dilution rate \( D \) as manipulated variable.

The dilution rate \( D \) in continuous fermentations is equal to the specific growth rate \( \mu \). In a turbidostatic culture the concentration of the growth limiting substrate \( S \) is usually much higher than the affinity constant \( K_s \), considering Monod’s kinetic. Therefore, the steady-state can be characterised by unlimited growth and dilution rates very close to \( \mu_{\text{max}} \) (Monod, 1949; Bryson, 1952). This allows the investigation of how environmental parameters such as temperature, pH value and concentrations of media components influence the maximum growth rate. The effects on \( \mu_{\text{max}} \) can be measured directly by means of changes in dilution rates. In cases of a positive influence of the environmental parameter on growth, dilution rate will rise, in case of a negative influence the dilution rate will decrease.

For studying the influence of CO\(_2\) on maximum specific growth rate of the microorganisms, the CO\(_2\) partial pressure of the inlet gas stream was varied. Though, the parameter that effects the response of the microorganisms to CO\(_2\) is the dissolved carbon dioxide concentration \( c_{\text{CO}_2(\text{dissolved})} \). The dissolved carbon dioxide concentration can be considered as the equilibrium between CO\(_2\) partial pressure of the inlet gas stream, the autogenuous CO\(_2\) that is generated and the CO\(_2\) that is consumed by the microorganisms.

The association/dissociation reactions of CO\(_2\) in water can be described by an equation of the form: \( \text{CO}_2(\text{dissolved}) + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{HCO}_3^- + \text{H}^+ \leftrightarrow \text{CO}_3^{2-} + \text{H}^+ \) (Mitz, 1979). The association/dissociation depends on the pH value of the solution. Among the chemical species in the liquid phase, only dissolved carbon dioxide \( \text{CO}_2(\text{dissolved}) \) contributes to the mass transfer of carbon dioxide across the gas–liquid interface.

For an estimation of the dissolved carbon dioxide concentration the following consideration is generally accepted:

\[
c_{\text{CO}_2(\text{dissolved})} = p_{\text{CO}_2(\text{dissolved})} L_{\text{CO}_2}
\]

where \( p_{\text{CO}_2(\text{dissolved})} \) is the partial pressure of CO\(_2\) in the liquid phase and \( L_{\text{CO}_2} \) is the solubility of carbon dioxide according to Henry’s Law, under the assumption of:

\[
p_{\text{CO}_2(\text{dissolved})} \approx p_{\text{CO}_2} = p_{\text{R}} y_{\text{CO}_2}
\]

where \( p_{\text{CO}_2} \) is the partial pressure of carbon dioxide and \( y_{\text{CO}_2} \) is the mole fraction of carbon dioxide at the gas–liquid interface.

Even though a precise mechanism for the effect of higher \( p_{\text{CO}_2} \) on microorganisms is still unknown (Isenschmid et al., 1995) has drawn a realistic hypothesis to explain the antimicrobial effect of carbon dioxide. Because of the unique properties of lipop- and hydrophilic, carbon dioxide could easily penetrate into the cell-membrane which leads to an increase of its fluidity and permeability. This so-called “anaesthesia effect” alters the characteristics of the membrane and destroys its essential domains thus leading to death of the cell. Another approach to explain the inhibiting effect of higher \( p_{\text{CO}_2} \) focuses on physiological effects. An accumulation of carbon dioxide in the cytoplasm can lower the internal pH by exceeding the buffer
capacity of the cell pool, leading to collapse of the pH gradient and the proton motif force. The intracellular bicarbonate and molecular carbon dioxide can interfere with a number of enzymes thereby inhibiting biochemical pathways. For instance decarboxylating enzymes are known to be inhibited by great carbon dioxide excess, braking the metabolic chain (Jones and Greenfield, 1982).

3. Material and methods

3.1. Strain

*C. glutamicum* wild-type ATCC 13032 was pregrown for 8 h on complex medium (yeast extract 5 g/L, peptone 5 g/L, d-glucose 10 g/L, NaCl 5 g/L, 30 °C, 250 ml shake flask, 25 mL filling volume, orbital shaker (Lab-Shaker LS-W, Adolf Kühner AG, Switzerland), shaking frequency of 280 rpm, shaking diameter of 50 mm) then washed twice in sterile NaCl solution (centrifugation 3000 × g, 4 °C, 15 min). The biomass was resuspended in mineral medium and inoculated into the bioreactor to result an optical density at 600 nm (Spectrophotometer Uvikon 922 A, Kontron Instruments, Italy) of approximately 1.5.

3.2. Mineral medium

The mineral medium was derived from Seletzky et al. (2006) and supplied with either d-glucose or l-lactate as sole carbon source: d-glucose or l-lactate 10 g/L, KH2PO4 1 g/L, K2HPO4 2 g/L, (NH4)2SO4 10 g/L, MgSO4·7 H2O 0.25 g/L, FeSO4·7 H2O 10 mg/L, MnSO4·H2O 10 mg/L, ZnSO4·7 H2O 1 mg/L, CuSO4·5 H2O 0.31 mg/L, NiCl2·6 H2O 0.2 mg/L, CaCl2 10 mg/L, Biotin 0.2 mg/L, 3,4-dihydroxybenzoic acid (protocatechuate) 30 mg/L. pH was adjusted to 7.0 by titration of NaOH solution.

3.3. Experimental setup of the turbidostat

Cultivations were carried out in a 1.5 L fermenter (Biostat-M, B. Braun, Melsungen, Germany) with a working volume of 800 mL furnished with four buffles of 0.14 m height and 0.014 m width and one 4 blade Rushton turbine of a diameter of 0.047 m and a blade height of 0.009 m. Aeration was kept constant at 1vvm and agitation speed was set to 1200 rpm. Dissolved oxygen partial pressure was measured with an AppliSense DO probe (Applikon, Netherlands). pH was measured with a sterilisable AppliSense pH probe (Applikon, Netherlands) and controlled with 2 M NaOH and 2 M H2SO4 solution. The amount of consumed acid and base was measured by two separate electronic balances (Kern KB 6500, Kern, Germany).

The optical density of the culture was used as signal for controlling the dilution rate of the continuous fermentation. Optical density (OD630 nm) was measured with a spectrophotometer (Skalar 6000, Netherlands) operating at a fixed wavelength of 630 nm with a flow cuvette of 0.5 mm optical path length (170 QS, Helma, Germany). The bubbles were seperated from the culture broth in a bubble seperator located in a by-pass, before the spectrophotometer. Bubble free culture broth as well as the bubbles were pumped back to the bioreactor. Online optical density was processed by a standard personal computer using the software LabView (V6.0, National Instruments, Germany). The cultures were started in batch mode and then switched to continuous turbidostatic mode, after a biomass concentration of more than 2 g/L CDW was achieved. For the batch and continuous cultures the same mineral medium was used. A PI-controller module inside LabView was applied to control the optical density by varying the pump rate of the feed solution. The consumption of the feed solution was measured by an electronic balance (EA60EDE-I, Sartorius AG, Germany) and the mean dilution rate D was calculated. The mean dilution rate was assumed to be equal to μmax.

3.4. Aeration

The inlet gas stream consisted of a defined mixture of air, pure carbon dioxide and oxygen. The mixture was prepared by three thermal mass flow controllers (EL-FLOW, Bronkhorst, Netherlands). Carbon dioxide was added to adjust a certain carbon dioxide partial pressure of the inlet gas stream up to 0.79 bar, while oxygen was added to keep the oxygen partial pressure of the gas mixture at 0.21 bar at any condition.

4. Results

Strain *C. glutamicum* ATCC 13032 was cultivated under turbidostatic conditions under carbon dioxide
Fig. 1. Example of the controlled parameter OD and the parameters pH, $p_{O_2}$ and feed consumption over time during the continuous culture of \textit{C. glutamicum} ATCC 13032 for growth on L-lactate as sole carbon source under stable turbidostatic steady-state conditions, a partial pressure of CO$_2$ of 0.14 bar and a mean dilution rate of 0.46 h$^{-1}$.

Partial pressures up to 0.81 bar with D-glucose and L-lactate as sole carbon sources. For every tested carbon dioxide partial pressure the culture was kept at least for 24 h under turbidostatic conditions. A mean dilution rate was calculated from the signal of the feed balance over time for a period of stable steady-state turbidostatic conditions. Stable steady-state turbidostatic conditions were achieved when OD was found to be constant over time.

Fig. 1 demonstrates a typical course of a continuous culture of \textit{C. glutamicum} under turbidostatic conditions. Under steady-state conditions the optical density of the culture broth was stabilized at OD$_{630\,\text{nm}}$ 0.4 (corresponding to 2.0 g/L CDW). The pH value was controlled at pH 6.85 and the oxygen partial pressure was oscillating between 20 and 30% air saturation. This oscillation is caused by varying feed rates of the OD control loop. The mean dilution rate for each $p_{CO_2}$ was calculated from the slope of feed consumption over time, as mentioned before.

Fig. 2 shows the results obtained with D-glucose as carbon source. For $p_{CO_2}$ up to 0.14 bar, $\mu_{max}$ was mostly constant around 0.58 h$^{-1}$. Between 0.14 and 0.27 bar, $\mu_{max}$ decreased to a $\mu_{max}$ of 0.52 h$^{-1}$. At very high $p_{CO_2}$ (0.81 bar), $\mu_{max}$ decreased down to 0.35 h$^{-1}$, which is 60% of $\mu_{max}$, compared to aeration with air.

Fig. 3 shows the dependency of $\mu_{max}$ of \textit{C. glutamicum} ATCC 13032 versus $p_{CO_2}$ for growth on L-lactate. For growth on L-lactate and aeration with pure air, $\mu_{max}$ was 0.38 h$^{-1}$ which is around 65% of $\mu_{max}$ compared to growth with air.

Fig. 2. Dependency of maximum specific growth rate vs. $p_{CO_2}$ for growth of \textit{C. glutamicum} ATCC 13032 on D-glucose as carbon source.

Fig. 3. Dependency of maximum specific growth rate vs. $p_{CO_2}$ for growth of \textit{C. glutamicum} ATCC 13032 on L-lactate as sole carbon source. Black diamonds: for step wised increase $p_{CO_2}$, white squares: for step wised reduction $p_{CO_2}$. 
to growth on D-glucose. Higher $p_{CO_2}$ up to 0.26 bar led to a significant increase of $\mu_{max}$ to around 0.47 h$^{-1}$ representing a 1.24-fold increase of $\mu_{max}$.

5. Discussion

The growth accelerating effect at higher $p_{CO_2}$ (Fig. 3) has not been reported before. The effect of $p_{CO_2}$ around 0.20 bar has been reported as growth inhibiting for a number of microorganisms before (Jones and Greenfield, 1982; Dixon and Kell, 1989; Onken and Liefke, 1989; McIntyre and McNeil, 1997; Debs-Louka et al., 1999; Spilimbergo and Bertucco, 2003). For $p_{CO_2}$ up to 0.32 bar, $\mu_{max}$ is still higher (0.45 h$^{-1}$) than for aeration with pure air. At very high $p_{CO_2}$ of 0.81 bar, $\mu_{max}$ is around 0.33 h$^{-1}$ which is 86% of $\mu_{max}$ achieved for aeration with pure air.

These effects might be explainable by the hypothesis that high $p_{CO_2}$ enhances the anaplerotic pathways of C. glutamicum. Growth on L-lactate and other carbon sources entering the central metabolism at the level of acetyl-CoA or pyruvate requires gluconeogenesis for providing the cells with 3-phosphoglycerate and with hexose and pentose sugars (Sauer and Eikmanns, 2005). The carboxylating reactions are important for gluconeogenesis and for the synthesis of precursor amino acids and nucleic acids. Regarding to the results, the hypothesis can be drawn that higher $p_{CO_2}$ enhance anaplerotic and maybe also gluconeogenetic reactions during growth on a substrate that requires gluconeogenesis. Obviously the influence of $p_{CO_2}$ on specific growth rate is dependent on the carbon source. During growth on D-glucose, a substrate that requires no major gluconeogenetic activity for the cells, no growth accelerating effects of higher $p_{CO_2}$ were detectable. Some authors found that during utilization of substrates with low number of C atoms, the importance of carboxylating reactions increase (Wendisch et al., 2000). The central metabolism of C. glutamicum growing on D-glucose is characterized by a low activity of the citric acid cycle (Kiefer et al., 2004) and the absence of glyoxylate cycle activity. In contrast, carbon sources such as acetic acid and probably also L-lactate lead to a high in vivo activity of the TCA cycle and the glyoxylate cycle whereas anaplerotic reactions and gluconeogenesis prevail (Wendisch et al., 2000). This might be the reason for the different growth behaviour of C. glutamicum on D-glucose and L-lactate under increased $p_{CO_2}$.

Some authors describe the use of continuous turbidostatic cultures as a tool to select the fastest growing microorganisms (Bryson, 1952). In our experiments we can exclude a growth accelerating effect caused by evolutionary selection of the turbidostat. Fig. 3 shows the reproducibility of our results for a stepwise increase (black diamonds) as well as for a stepwise reduction of the $p_{CO_2}$ (white squares). In case of an evolutionary selection of microorganisms with higher growth rate, an acceleration of $\mu_{max}$ could occur over time and both curves would not fit to each other as in Fig. 3.

6. Conclusion

The influence of increased $p_{CO_2}$ on growth of C. glutamicum ATCC 13032 was demonstrated under continuous turbidostatic steady-state conditions for the growth on D-glucose and L-lactate as sole carbon source. The continuous turbidostatic culture system has proven to be a valuable tool for the characterisation of the reaction of microorganisms to increased $p_{CO_2}$. It was shown, that $p_{CO_2}$ of about 0.2–0.3 bar does not inhibit the growth of the microorganism. In contrary, it is enhancing the maximum growth rate of C. glutamicum on L-lactate as carbon source up to the 1.24-fold comparing to aeration with normal air. Even at very high $p_{CO_2}$ of 0.81 bar, 86% of maximum growth rate on L-lactate, was achieved, compared to aeration with normal air. Thus, high hydrostatic pressures in industrial scale fermenters, pressure fermentations and high cell density cultures do not always result in disadvantageous culture conditions for the microorganisms. It is clearly shown, that the reaction of microorganisms to increased $p_{CO_2}$ strongly depends on the carbon source. The response of microorganisms has to be investigated as part of the process design. It is believed, that this method could be very helpful for the investigation of the impacts on anaplerotic reactions. For future investigations, growth and product formation by production strains or genetically modified mutants under different carbon dioxide concentrations and turbidostatic conditions might help to identify the role of the various anaplerotic pathways. Another interesting approach will be to investigate influence of carbon dioxide on metabolic fluxes in dependency to the $p_{CO_2}$. 

References


