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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

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Oxygen limitation is a pitfall during screening for industrial strains

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Abstract Oxygen supply is a key parameter in aerobic fermentation processes like the industrial production of amino acids. Although the oxygen transfer rate (OTR; or the volumetric oxygen transfer coefficient k_La) is routinely analyzed by engineers during stirred tank fermentations, it is often not taken into account by biologists conducting screening experiments in shake flasks. To show the importance of knowing how to avoid oxygen transfer limitations during primary screenings, *Corynebacterium glutamicum* ATCC 13032 (wild-type strain) and DSM 12866 (lysine-producing strain) were cultivated in shake flasks with different culture liquid volumes and under different shaking conditions. With the Respiration Activity Monitoring System, the OTR was determined quasi-continuously. Optical density as well as concentrations of lysine and byproducts (lactate, acetate, succinate) were determined off-line and correlated with the OTR signal. From the results, design criteria for improved screening in shaken bioreactors that help to avoid selection of suboptimal strains during early process development steps can be derived. Finally, the suitability of DSM 12866 as a strain for industrial processes with a high space–time yield is discussed.

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Introduction

Aeration and oxygen supply are parameters well-known by biochemical engineers but are also of increasing interest to biologists due to the interdisciplinary character of biotechnology, as well as the rapid progress in applied biosciences (Rainer 1990). The number of fermentations that are run worldwide in shaken bioreactors is estimated to be several millions per year. These experiments are in many cases conducted by researchers who have little knowledge in biochemical engineering. An in-depth understanding of molecular biology and physiology of industrial relevant organisms is essential for the development of genetically engineered production strains because the metabolic network of an organism is an extremely complex system. In reality, however, most of the metabolic reactions are rather inactive and only selected fluxes dominate the metabolism, whereas the regulation of these fluxes depends strongly on cultivation conditions (Fischer and Sauer 2003; Almaas et al. 2004). In particular, it is generally known that the central metabolism reveals significant differences under fully aerobic and oxygen-limited conditions (Dominguez et al. 1993; Varma et al. 1993). Correlations found under nonrepresentative conditions of the screening system might prove to be wrong or have an opposite trend under production conditions (Freyer et al. 2004). Hence, a biotechnologist has to know the biochemical engineering conditions of an experiment to be able to consider these parameters during interpretation of results. This avoids the selection of suboptimal strains during the early steps of bioprocess development. The selection of a suboptimal strain during the primary screening would have a negative influence on later process development steps and, in many cases, cannot be compensated (Büchs 2001). To substantiate these assertions, two *Corynebacterium glutamicum* strains were examined under different process conditions in shake flasks using automated monitoring of the oxygen transfer rate (OTR), which is a key parameter of aerobic fermentation processes in any scale.

Materials and methods

Strains and growth medium

C. glutamicum ATCC 13032 (wild-type strain) and DSM 12866 (lysine-producing strain; Kreutzer et al. 2001) were used as demonstration organisms. The shake flask growth medium had the following composition per liter: 55 g glucose·H_2O (Merck, Darmstadt, Germany), 5 g corn steep liquor (Roquette, Lestrem, France), 20 g morpholino propane sulfonic acid (Serva, Heidelberg, Germany), 25 g $(NH_4)_2SO_4$, 0.1 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 10 mg $CaCl_2 \cdot 2H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 5 mg $MnSO_4 \cdot H_2O$ (all from Merck), 15 mg 2% D-biotin dextrin sorbic acid (Roche, Basel, Switzerland), 0.2 mg thiamine·HCl (Merck), 25 g $CaCO_3$ (Merck). Glucose and vitamins were dissolved and sterile filtered. $CaCO_3$ was autoclaved separately as dry powder. Other components were dissolved and autoclaved after the pH had been adjusted to 7 with 10% NaOH (Merck). Finally, all components were aseptically united. It is worth mentioning that most of the $CaCO_3$ is not soluble. This excess of $CaCO_3$ is not osmotically active and serves as a buffer supply.

Fermentation conditions and analytics

For online determination of the OTR in shake flasks, the Respiration Activity Monitoring System (RAMOS, HiTec Zang, Herzogenrath, Germany) was applied. This apparatus has been described elsewhere in detail (Anderlei and Büchs 2001; Anderlei et al. 2004). It measures the decrease of oxygen partial pressure in the gas phase of eight closed 250-ml shake flasks with an oxygen sensor mounted in the neck of each flask. From the slope of the oxygen partial pressure curve, the system calculates the OTR. To avoid oxygen depletion, the flasks are automatically flushed with ambient air repetitively. In the present work, a measuring and flushing interval of 30 min was selected, i.e., the measurement was performed quasi-continuously. The repeating intervals were controlled in such a way that the resulting average oxygen partial pressure in the gas phase of a measurement flask was equal to the partial pressure in a conventional shake flask sealed with a normal sterile plug. Hence, conventional unbaffled shake flasks were used for several parallel experiments to enable easy manual sampling for off-line analytics without the need to intervene in the RAMOS process. Flasks were shaken on an orbit with a 5-cm diameter at two shaking frequencies (300 and 200 rpm). Two culture volumes (20 and 10 ml) were used, and the cultures were inoculated directly from glycerol stocks. Incubation temperature was 33 °C throughout the study. For each RAMOS experiment, five parallel Erlenmeyer flasks were run at equal shaking frequency, with equal inoculum and with equal filling volume to enable noninvasive off-line analytics. In addition, the initial medium at the beginning of each experiment was analyzed. The optical density was determined in a standard 1-cm cell photometer at 660 nm after diluting part of the samples in 0.1 N HCl (Merck) to dissolve all $CaCO_3$ and to ensure a value in the linear range of the photometer. All other analytics were conducted after sample centrifugation and supernatant filtration. Glucose was measured on a glucose analyzer (YSI 2700 Select, Yellow Springs Instruments, Yellow Springs, OH, USA). Lactate, acetate, and succinate were determined by high-performance liquid chromatography using a RP-8 column and a flow rate of 1.0 ml min^{-1}. The mobile phase composition was: 205 ml phosphate buffer [0.1 M $NH_4H_2PO_4$ and 0.1 M $(NH_4)_2HPO_4$], 100 ml H_2O, and 440 ml acetonitrile. Lysine was determined on an amino acid analyzer (Biochrom 20, Amersham Pharmacia Products, Piscataway, NJ, USA) and converted to lysine·HCl equivalents.

Results

The results from OTR measurements and from offline analyses are shown in Figs. 1, 2, and 3. At the beginning of each experiment, the OTR rises until the maximum oxygen transfer capacity (Maier and Büchs 2001) of the shake flask (depending on shaking frequency and culture volume) is reached (Figs. 1, 2 and 3a). At this point the OTR curve forms a plateau (Anderlei et al. 2004; Kensy et al. 2005) until all carbon sources are exhausted and the OTR rapidly drops. This defines the end of a process. DSM 12866 does not reach the maximum oxygen transfer capacity when shaken at 200 rpm with 10 ml liquid volume (Fig. 3b) or when shaken at 300 rpm. Hence, the fermentation results for this strain shaken at 300 rpm are equal to those of Fig. 3b (data not shown). At the very beginning of each fermentation, the initial lactate (an ingredient of corn steep liquor) is consumed. After that, the cells change their metabolism for growth solely on glucose, which is

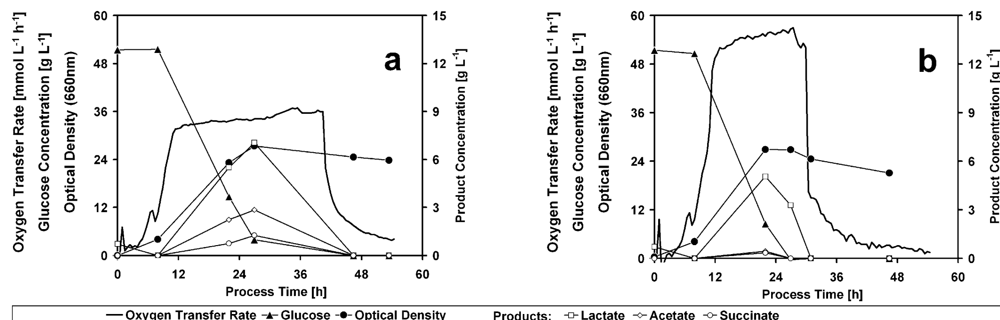

Fig. 1 *C. glutamicum* ATCC 13032 shake flask fermentations at a shaking frequency of 300 rpm. Culture liquid volume is **a** 20 ml and **b** 10 ml

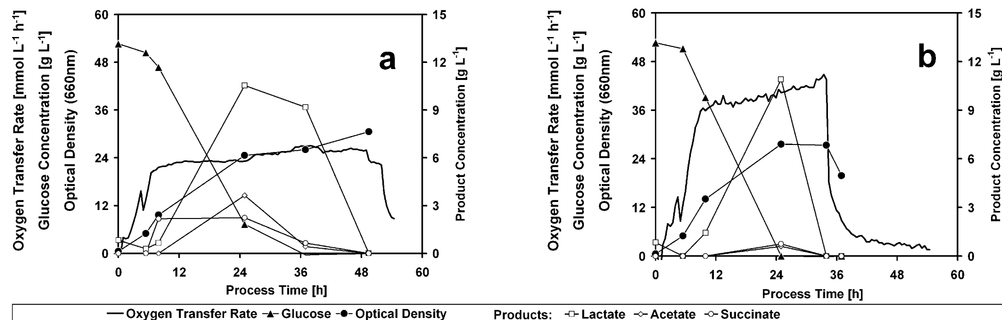

Fig. 2 *C. glutamicum* ATCC 13032 shake flask fermentations at a shaking frequency of 200 rpm. Culture liquid volume is **a** 20 ml and **b** 10 ml

indicated in all fermentation experiments by a tiny OTR peek after approx. 6 h (Figs. 1, 2 and 3). Then the cells consume glucose. Later, ATCC 13032 generates lactate, acetate, and succinate while cultivated under oxygen limitation (Figs. 1 and 2). As the growth medium contains an excess of $CaCO_3$ salt, the acidic byproducts do not significantly change the pH of the broth; therefore, their presence does not have a significant negative effect on cell physiology. The lower the oxygen transfer capacity, the higher is the formation of byproducts and the longer is the processing time (Figs. 1 and 2). After exhaustion of all carbon sources, the processes terminate. The total oxygen transfer, which is defined by the integral under the oxygen transfer rate function, reached 1.2 mol l^{-1} during the four ATCC 13032 processes and 0.8 mol l^{-1} during both DSM 12866 fermentations. DSM 12866 generates L-lysine during growth (Fig. 3) according to a type-(I) fermentation kinetic (Gaden 1959). Neither acetate nor succinate was detectable during DSM 12866 fermentations (data not shown). Glucose utilization rate is much lower for DSM 12866 than for ATCC 13032, which correlates with different slopes of the OTR curves before reaching the maximum oxygen transfer capacity. All these results clearly show that much more information can be obtained by the quasi-continuous OTR signal than by the graphs drawn from discontinuous optical density or glucose concentration measurements.

Discussion

DSM 12866 was derived from ATCC 13032 by several rounds of mutagenesis and screening (Kreutzer et al. 2005). The exact screening conditions have not been published, but during screening, the maximum oxygen transfer capacity was probably not above 30 mmol l^{-1} h^{-1} (corresponding volumetric oxygen transfer coefficient k_La 150 h^{-1}) because this limit can be expected for standard shaken flasks (Maier and Büchs 2001; Wittmann et al. 2003). On the other hand, capacities that are several times higher have been applied for decades in large-scale stirred vessels (Van't Riet 1979; Moser 1988) and can be reached even in microtiter plates shaken under appropriate process conditions (Kensy et al. 2005). Growth limitation is known to be in accord with high productivity in certain lysine producing strains (Eggeling et al. 1998). A long processing time, however, is an unfavorable cost factor for industrial processes, especially for bulk products like feed grade amino acids. DSM 12866 is difficult to apply in processes with high space–time yield. In Fig. 3, the slopes of OTR graphs at the beginning of fermentations is significantly lower compared to the wild type (before oxygen limitation), especially after the initial lactate is exhausted. Furthermore, the total oxygen transfer of DSM 12866 amounts only two-thirds of the value reached during fermentations of ATCC 13032. These observations indicate an altered metabolic activity of DSM 12866. To some extent, this could be explained by the fact that this strain generates lysine instead of biomass. In addition, there might be one or more undesirable mutations that reduce glucose uptake or glucose utilization of the strain. Such mutations might have been selected accidentally during screening between mutagenesis steps because they also reduce formation of lactate, acetate, and succinate under oxygen-limited conditions. Therefore, such mutants with low metabolic activity might have been selected predominantly due to wrong screening conditions (Büchs 2001). Generation of byproducts might have been thought to be disadvantageous during screening in shake flasks, although it was mainly caused by oxygen limitation and not to be

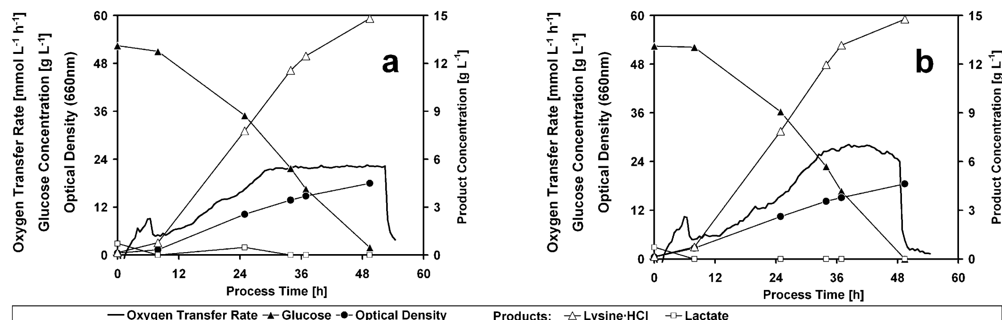

Fig. 3 *C. glutamicum* DSM 12866 shake flask fermentations at a shaking frequency of 200 rpm. Culture liquid volume is **a** 20 ml and **b** 10 ml

expected in well-aerated stirred tanks. Moreover, if the screening during breeding of DSM 12866 was conducted in a weakly buffered medium, the acidic byproducts might have caused a severe drop of pH, and thus, poor experimental results might have been encountered. Long processing times could not have been realized as a disadvantage because shorter processing times were not possible in shaken flasks under oxygen-limited conditions, even for the wild-type strain (Fig. 2a). On one hand, high glucose uptake and utilization requires high activity in the respiratory chain. On the other hand, oxygen limitation reduces cell respiration. As a consequence, intracellular pyruvate is accumulated and converted to anaerobic fermentative byproducts. However, certain mutations, resulting in lower glucose conversion rate to pyruvate, could avoid byproduct formation under oxygen limitation but, at the same time, prevent higher fluxes from glucose via pyruvate to lysine even under high aeration rates in stirred tank fermentors. Hence, the development of a cost-effective aerobic production process based on such a strain became rather difficult. These considerations led to the conclusion that strains like DSM 12866 might have never been selected during screening as potential precursors for production strains if the maximum oxygen transfer capacity would had been taken into account during the design of screening experiments and the interpretation of screening results.

Approaches to rational strain design have recently gained more and more importance in industrial strain development (Hüser et al. 2005; Peters-Wendisch et al. 2005; Wittmann and de Graaf 2005) and tend to substitute classical breeding by random mutagenesis and selection. Nevertheless, even such studies should be carried out solely under sufficient oxygen supply to avoid the design of experiments with poor correlation to industrial aerobic production processes. Last but not least, the OTR (or k_La) is an essential part of characterizing bioreactors employed for aerobic fermentations and a useful tool for subsequent scale-up arrangements (Rainer 1990).

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