Effect of Oxygen Limitation and Medium Composition on *Escherichia coli* Fermentation in Shake-Flask Cultures

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Shake-flask cultures are widely used for screening of high producing strains. To select suitable strains for production scale, cultivation parameters should be applied that provide optimal growth conditions. A novel method of measuring respiratory activity in shake-flask cultures was employed to analyze *Escherichia coli* fermentation under laboratory conditions. Our results suggest that the length of fermentation, choice of medium, and aeration do not normally satisfy the requirements for unlimited growth in shake flasks. Using glycerol rather than glucose as a carbon source greatly reduced the accumulation of overflow and fermentative metabolites when oxygen supply was unlimited. A rich buffered medium, Terrific Broth (TB), yielded 5 times more biomass compared to LB medium but also caused oxygen limitation in standard shake-flask cultures at shaking frequencies below 400 rpm. These results were used to optimize the production of benzoylformate decarboxylase from *Pseudomonas putida* in *E. coli* SG13009, resulting in a 10-fold increase in volumetric enzyme production. This example demonstrates how variation of medium composition and oxygen supply can be evaluated by the measurement of the respiratory activity. This can help to efficiently optimize screening conditions for *E. coli*.

Introduction

Complex media such as LB (Luria Bertani) medium and TB (Terrific Broth) have been successfully used to grow *Escherichia coli* in small-scale cultures. Since the development of recombinant DNA technology and the production of heterologous proteins, it has become increasingly important to optimize growth and expression (*1*−*4*). However, the physiological implications of small-scale culture conditions, as opposed to fermentation processes in stirred bioreactors, have not been studied in detail (*5*). Successful expression of proteins in a shake flask cannot easily be up-scaled, if cultivation parameters are not well characterized. Moreover, unfavorable conditions during screening for a production strain may lead to selection of a strain that is unsuitable for large-scale fermentations. Especially, the supply of oxygen as a key parameter for growth of facultative anaerobes has only been monitored indirectly in shake flasks (*6*). Because the expression of more than 200 genes by *E. coli* is dependent on the availability of oxygen (*7*), it is important to control aeration (*2*). Here we provide data on the respiratory activity of *E. coli* JM109 in shake-flask cultures using a novel method of measuring oxygen transfer rates (OTR) and carbon dioxide transfer rates (CTR) (*8*, *9*). This system measures the uptake of oxygen by analyzing the depletion of oxygen in the gas phase of the shake flask. Moreover, the system uses a pressure sensor to facilitate the measurement of the CTR. The liquid phase of the flask remains undisturbed, resulting in culture conditions identical to those of normal shake flasks. Experiments were performed with the aim of establishing a better understanding of the effects of basic cultivation methods and to assess means of improving these. Respiratory activity is a very sensitive parameter and can yield highly valuable information about the carbon metabolism of the cultured cells. Primary metabolism of *E. coli* responds quickly to oxygen limitation, changes in pH, and acetate concentration (*10*−*12*), which hinder optimal growth conditions over a long culture period. This explains the discrepancy between cell densities obtained in stirred bioreactors as compared to shake-flask cultivation. Many modifications to the LB medium have been described with the aim of increasing cell yield in shake-flask cultures (*13*−*18*). These include supplementing both fermentable and nonfermentable carbon sources and the addition of buffers. The use of glycerol as a nonfermentable carbon source and phosphate buffer is also the main difference between LB medium and TB medium (see Table 1). In this study *E. coli* was cultivated in different complex media. In each case the influence of oxygen supply, carbon source (glucose, glycerol), and pH buffer on respiratory activity over time was investigated. Optimized culture conditions were used to overexpress a variant of benzoylformate decarboxylase (BFD), from *Pseudomonas putida* in *E. coli* SG13009 (*19*). It is the aim of this work to demonstrate a new methodology for the investigation of small-scale cultures, applying a new device for on-line measurement of the OTR and the CTR. Using this system, the influence of medium composition and oxygen supply on the metabolic activity was

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Table 1. Composition of Media

component	LB (buffered)[a]	LB-glucose (buffered)[a]	LB-glycerol (buffered)[a]	TB
tryptone	10 g/L	10 g/L	10 g/L	12 g/L
yeast extract	5 g/L	5 g/L	5 g/L	24 g/L
glucose		10 g/L		
glycerol			10 g/L	5 g/L
salts	5 g/L NaCl[a]	5 g/L NaCl[a]	5 g/L NaCl[a]	12.54 g/L K_2HPO_4 2.31 g/L KH_2PO_4

[a] Buffered versions of LB, LB-glucose, and LB-glycerol were prepared by replacing NaCl with 89 mM phosphate buffer (12.54 g/L K_2HPO_4, 2.31 g/L KH_2PO_4), adjusted to pH 7.

systematically studied, and critical culture parameters for growth and protein expression in shake flasks were identified.

Methods

Bacterial Strain, Media, and Culture Conditions. *E. coli* JM109 (ATCC 53323, DSMZ, Braunschweig, Germany) was used to study the effects of growth conditions. *E. coli* SG13009 (Qiagen, Hilden, Germany) was used to test heterologous protein expression under optimized growth conditions. Cloning of this strain has been described (*19*). This strain was transformed with the vector pKK 233-2 (Pharmacia, Uppsala, Sweden) containing a mutated gene for benzoylformate decarboxylase (BFD) from *P. putida* and hence producing a variant of BFD with a single amino acid exchange (L476Q).

Terrific Broth (TB medium) (*20*), Luria Bertani medium (LB medium) (*20*), and variants thereof were prepared accoring to Table 1 using tryptone (Difco from Becton Dickinson, Franklin Lakes, USA), yeast extract (Difco), glucose, glycerol, NaCl, K_2HPO_4, and KH_2PO_4 (Merck, Darmstadt, Germany). The pH of LB medium, LB-glucose, and LB-glycerol was close to 7 without adjustment. Phosphate salts, carbon sources, and complex medium compounds were autoclaved separately. For cultivation of *E. coli* SG13009, 100 μg/mL ampicillin and 50 μg/mL neomycin were added to the medium. BFD production was induced 3.5 h after inoculation with 1 mmol/L IPTG.

Shake flasks with a nominal volume of 250 mL and a cotton plug were filled with either 10 or 20 mL of medium inoculated by a LB preculture (1/100, v/v). Cultures were grown at 37 °C on an orbital shaker at a shaking frequency of 200 or 400 rpm with a shaking diameter of 5 cm (Lab-shaker, Kühner AG, Birsfelden, Switzerland). Up to six different media were tested in parallel and inoculated from the same preculture. For each culture condition 10−15 shake flasks were filled with the same volume of inoculated medium. Additionally one adapted shake flask was used for measuring the respiration activity (see below). For each sampling point, one of the flasks was removed from the shaker and used directly for measurement of ammonium concentration, pH, and OD and for HPLC analysis. Flasks removed from the shaker for sampling were not reused.

Oxygen and Carbon Dioxide Transfer Rate Measurements. The respiratory activity of a shake-flask culture corresponds to the oxygen transfer rate (OTR) between the gas and the liquid phase. The OTR was measured using a respiration activity monitoring system (RAMOS, Hitec Zang, Herzogenrath, Germany). The function of this device (*8*) is based on analyzing the oxygen concentration in the gas headspace of the shake flask with an oxygen sensor. During a measuring phase (12 min) the flask is entirely sealed and the depletion of oxygen can be measured. The slope of the depletion corresponds to the OTR. Subsequently, a rinsing phase (18 min), during which a controlled flow of air is lead through the shake flasks, restores the original gas equilibrium. For this purpose, inlet and outlet valves that are connected to the top of each flask are opened. The air flow rate is controlled in such a way that the average gas concentration in the headspace of the measuring flask and in the normal shake flasks with cotton plugs is equivalent. The cycle of measuring and rinsing phases is continually repeated. The carbon dioxide transfer rate (CTR) was calculated using the OTR and the change of pressure during the measuring phase (*9*).

Unlimited oxygen supply for *E. coli* in LB-glycerol and TB medium was attained by increasing the shaking frequency to 400 rpm and supplying the flasks of the RAMOS device with a mixture of oxygen and air; oxygen partial pressure was adjusted to 4.2×10^4 Pa (approximately 42% O_2, twice the concentration of air) by mass flow controllers (201C, Bronkhorst, Ruurlo, The Netherlands). Together, the doubling of the oxygen concentration and the shaking frequency facilitates a 4-fold increase in maximum oxygen transfer capacity (*3*).

Ammonium Measurement. Ammonium concentration was measured using an ammonium-sensitive electrode (Cole-Parmer Instrument Company, IL, USA). A calibration curve using NH_4Cl solutions of 0.5, 2, and 10 mmol/L was generated shortly before each measurement. Values were verified semiquantitatively with ammonium-sensitive test-strips ("Merquoquant", Merck, Darmstadt, Germany).

pH Measurement. In some experiments the pH was continuously monitored using a pH electrode (405-DPAS-SC-K8S, Mettler Toledo, Giessen, Germany) positioned so that it came into contact with the culture liquid with each agitation. The electrode was assembled to an adapted RAMOS measuring flask and autoclaved for 20 min in place. Values were verified by offline pH readings of parallel culture samples (Inlab 490, Mettler Toledo).

Cell Density Measurement. Cell density was monitored by measuring the optical density (OD) at a wavelength of 600 nm with a spectrophotometer (Uvikon 922 A, Kontron Instruments, Milano, Italy) using 10 mm cuvettes. Samples were diluted (9 g/L NaCl) to keep OD readings in the linear range between 0.03 and 0.3. For the measurements, 9 g/L NaCl was used as a blank; the OD of sterile media was subtracted from the measured OD to obtain the cell density.

Substrate and Metabolite Determinations. Concentrations of the medium components glucose and glycerol and the fermentation products acetate and lactate were measured by HPLC equipped with an Aminex HPX 87 H column (Bio-Rad, Hercules, USA). The column was eluted isocratically with 1 mmol/L H_2SO_4; 20 μL was injected per sample. Peaks were detected by recording the refractive index (Shodex RI-71, Showa

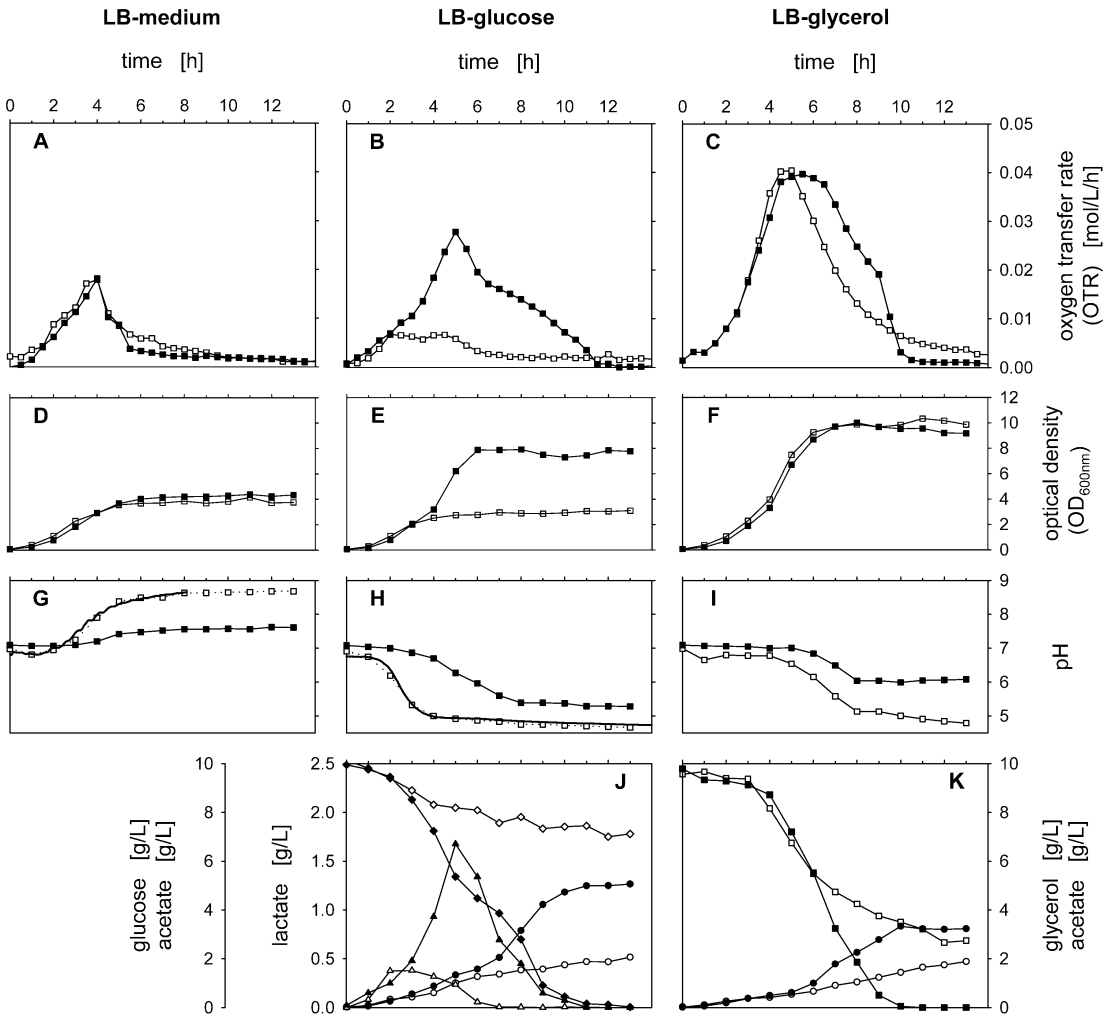

Figure 1. Growth parameters of *E. coli* JM109 in modified LB media. Filled symbols: phosphate buffered media. Open symbols: unbuffered media. (A−C) Respiratory activity. The very low respiratory activities in LB and LB-glucose lead to unlimited oxygen supply (A and B), whereas LB-glycerol cultures are oxygen-limited at OTR = 0.04 mol/L/h. (D−F) Optical density. (G−I) pH changes. The pH for LB and LB-glucose was measured online (solid lines in D and E) and verified by pH readings of parallel cultures (□). (J, K) Substrate and metabolite concentrations: glucose (◇ and ◆), lactate (▲ and △), acetate (○ and ●), and glycerol (■ and □). Filling volume 10 mL, shaking frequency 200 rpm, shaking diameter 50 mm.

Denko, Düsseldorf, Germany) or the UV absorbance at 220 nm (UV/VIS Detector 170D, Dionex, Germering, Germany).

Benzoylformate Decarboxylase (BFD) Activity Test. Intracellular concentrations of BFD were determined using a coupled decarboxylase assay after lysozyme cell disruption (*21*). Cell pellets from 3 mL of culture medium were stored at −20 °C and subsequently resuspended in extraction buffer (50 mol/L K_3PO_4, 5 mmol/L $MgSO_4$, 0.5 mmol/L thiaminediphosphate, pH 7). These samples were then ultrasonicated for 5 min and centrifuged at 10 000*g* for 15 min. Supernatants were prediluted at 1/100 in reaction buffer (same as extraction buffer but pH 6). The decarboxylase reaction contained the following components, diluted in reaction buffer: 5 mmol/L benzoylformate, 0.5 mmol/L NADH, and 0.25 mg/mL horse liver ADH (Sigma, Taufkirchen, Germany). Each assay was performed at 30 °C in a cuvette with a 1 mL reaction volume using 50 μL of diluted cell extract. Enzyme activity was measured by following the depletion of NADH photometrically at 340 nm for 90 s. Purified BFD from *P. putida* was used as a standard (*21*). The protein content of the cell lysates and the concentration of purified BFD were determined according to the method of Bradford. The proportion of BFD in the cell extract was calculated by the quotient of the specific activity in the cell extract and the specific activity of purified BFD.

Results and Discussion

LB Medium. *E. coli* grown in normal LB medium results in an increasing respiratory activity over 4 h (Figure 1A, □) reaching an oxygen transfer rate (OTR) of only 0.02 mol/L/h. Under the applied shaking conditions (filling volume 10 mL, shaking frequency 200 rpm) the maximum oxygen transfer capacity is 0.04 mol/L/h (*3*); therefore oxygen limitation is not the cause of the low respiration activity. Because LB medium consists mainly of nitrogen-containing complex compounds (Table 1), the cells are forced to use these as energy sources. This results in the production of ammonium with a maximum concentration of 4 mmol/L at 5 h (data not shown), thereby increasing the pH to 8.4 (Figure 1G). Stripping of carbon dioxide (measured by the carbon dioxide transfer rate, CTR data not shown) after main respiration stops further increases the pH slightly, finally reaching pH 8.7. Buffering LB medium with 89 mmol/L phosphate limits this pH increase to pH 7.6. However, this has practically no effect on the respiratory activity (Figure 1A, ■) or on final cell density (Figures 1D and 2). This suggests that the growth-limiting factor of LB

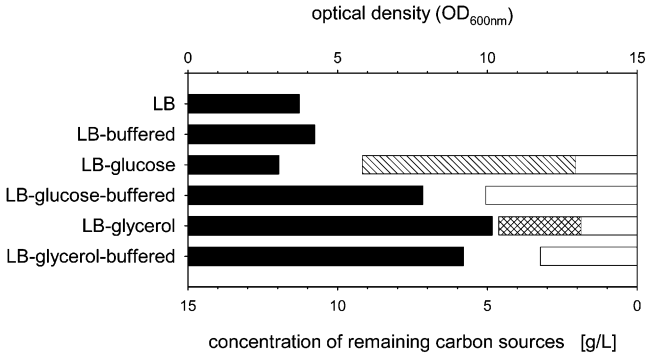

Figure 2. Summary of products from LB cultures in stationary phase (14 h in Figure 1): (black bars on left) cell density; (hatched bar) remaining glucose; (crosshatched bar) remaining glycerol; (open bars) acetate.

medium is not the buffer capacity of the medium but the amount of available carbon sources.

LB-Glucose. Supplementing LB medium with glucose under the same shaking conditions (filling volume 10 mL, shaking frequency 200 rpm) led to a decrease of respiratory activity (Figure 1B, □) corresponding to a rapid drop in pH to below 5 within 4 h (Figure 1H). Overflow metabolism, resulting in the production of lactic and acetic acid (Figure 1J), was responsible for this adverse effect. The small amount of glucose that was metabolized was primarily converted to acetic acid. The final cell density was lower than that formed in LB medium (Figures 1D, 1E, and 2). The maximum ammonium concentration was also two times lower than that in LB medium, reaching only 2 mmol/L (data not shown).

Because high concentrations of acetate and lactate are toxic especially at low pH (*22*, *23*), LB-glucose was buffered with phosphate in the same way as described above for LB medium and used with the same shaking condition (filling volume 10 mL, shaking frequency 200 rpm). Buffering significantly improved the respiratory activity, but the OTR did not reach the oxygen transfer capacity of the flask of 0.04 mol/L/h (Figure 1B, ■). Final cell density was increased 2-fold compared to that in LB medium or LB-glucose without buffer (Figure 2). Glucose was completely consumed within 12 h. However, overflow metabolism still occurred. Lactate was produced intermediately but reused again (Figure 1J), while acetate accumulated to 5 g/L, accounting for almost 50% of the original carbon source added to the medium.

LB-Glycerol. As a result of the production of overflow metabolites, the yield of biomass from added glucose was found to be largely diminished. Therefore glucose was substituted by glycerol, in both the buffered and the nonbuffered media.

Nonbuffered LB-glycerol reached high OTRs (Figure 1C) and a higher OD (Figures 1F and 2) than the other LB medium modifications. Also, a drop in pH (Figure 1I) due to acetate production (Figure 1K) was observed, but this occurred 4 h later than in the corresponding unbuffered glucose culture, thus maintaining a longer growth phase. Using this medium, ammonium was also produced to a maximum concentration of 2.5 mmol/L (data not shown) while some glycerol remained in the medium (Figures 1K and 2). No production of lactate was observed using LB-glycerol.

Buffering of LB-glycerol led to a complete consumption of glycerol (Figures 1K and 2). However, this was associated with a higher production of acetate, therefore not improving cell density. The start of the drop in pH due to an increased production of acetate corresponded to the time point of the maximum OTR. Here, the culture reached the maximum transfer capacity of 0.04 mol/L/h at these shaking conditions (filling volume 10 mL, shaking frequency 200 rpm). The resulting flat course of the OTR is typical for oxygen limitation (*8*).

Oxygen Limitation in LB-Glycerol. Since buffering of LB glycerol did not increase cell density, the influence of oxygen supply on unbuffered LB-glycerol cultures was tested by altering aeration parameters (unlimited oxygen supply − 400 rpm, 42% oxygen, and filling volume of 10 or 20 mL; limited oxygen supply − 200 rpm, 21% oxygen (air), and filling volume of 10 or 20 mL). Respiration of these cultures is strongly dependent on shaking frequency, oxygen concentration, and medium volume, thus demonstrating oxygen limitation (Figure 3A, note different scale of the OTR axis). The maximal respiratory activity (0.025 and 0.045 mol/L/h) of the cultures at 200 rpm corresponds to the oxygen transfer capacity of the shake flasks at the respective filling volumes (*3*, *24*). The finding that the respiratory profiles of cultures incubated at 400 rpm and 42% oxygen do not depend on filling volume suggests that these cultures are not oxygen-limited. Oxygen-limited cultures using LB-glycerol had a final pH of 4.8 (Figure 3E) and a final acetate concentration of 1.6 g/L (Figures 3I and 4), whereas oxygen-unlimited cultures had a final pH of 7.3 and no acetate was detected (Figure 4). Therefore, in the case of LB-glycerol, acetate production is a consequence of oxygen limitation. The combination of low pH values with the production of acetate results in a reduction of growth and glycerol consumption (Figures 3C, E, G, I and 4). As a consequence, cell yield in LB-glycerol is optimal under oxygen-unlimited conditions (Figure 4).

TB Medium. TB medium is a complex medium containing a high amount of yeast extract and tryptone. As opposed to LB medium it is buffered with 89 mM phosphate and contains glycerol as a carbon source. TB medium is similar to buffered LB-glycerol but contains a smaller amount of glycerol and a higher amount of complex compounds. Using TB medium, *E. coli* JM109 reached its maximum respiratory rate of 0.12 mol/L/h (Figure 3B) but required oxygen-enriched air, high shaking frequencies, and low filling volumes. The plateaus of respiratory activity of cultures grown at low shaking speed (200 rpm) clearly indicate oxygen limitation (*8*). A large amount of glycerol is converted to acetate (Figure 2F, H). When glycerol is depleted (Figure 3H), cells started to grow on acetate. This transition can be followed by the short period of reduced respiration after 6 and 7 h, respectively, depending on the filling volume (Figure 3B). A lag phase in the OD curve of the 20 mL culture also indicates the change of the carbon source (Figure 3D). Oxygen limitation results in a longer culture time but does not cause a loss in cell yield as was observed in the case of LB-glycerol. With final cell densities of OD_{600} = 20 (Figure 3D), the performance of TB medium was far superior as compared to the other media tested. Neither glycerol nor acetate was found in the cultures after the drop of respiratory activity, indicating that the amount of carbon source and complex compounds are well balanced in this medium. At the peak of acetate concentration the phosphate buffer in TB medium does not allow the pH to fall below 6.3 (Figure 3F). At this pH, growth of *E. coli* in a complex medium is inhibited but not prevented by the observed acetate concentration (*10*). The comparison between TB medium and the similar LB-glycerol indicates that the balance between glycerol and complex compounds is critical in oxygen-limited cultures.

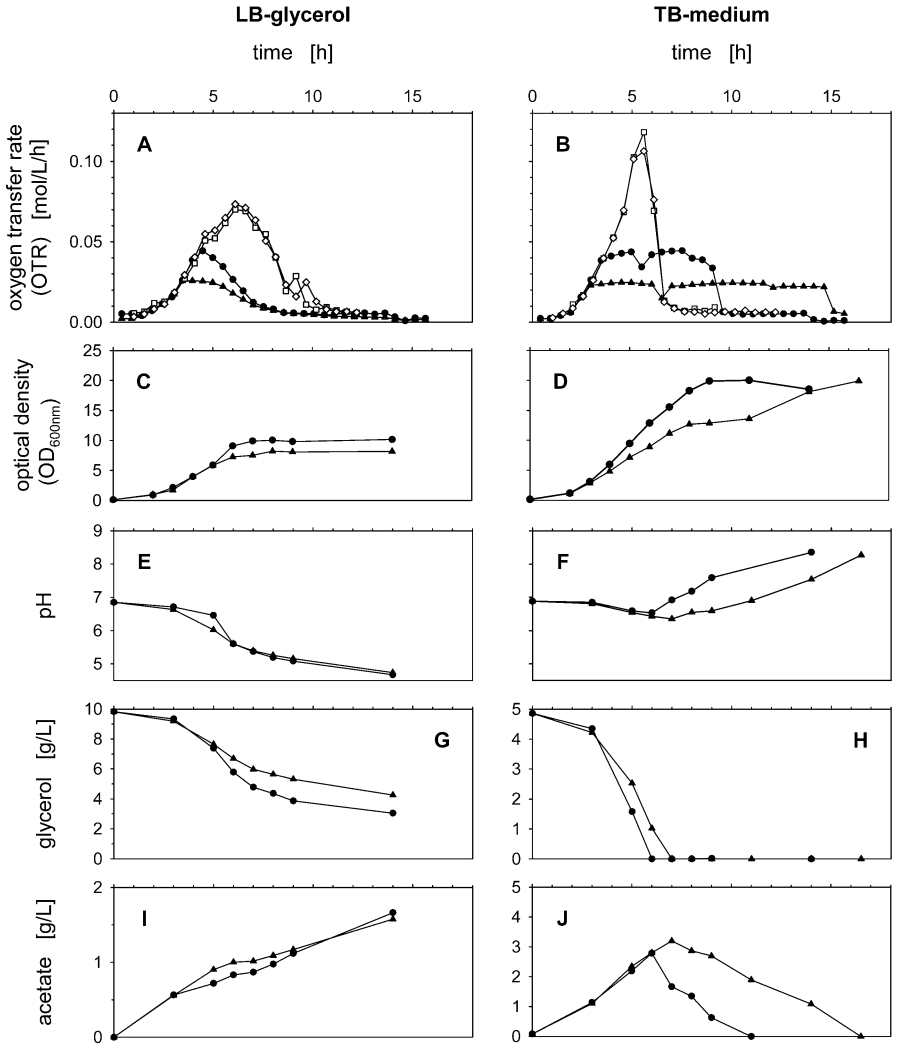

Figure 3. Oxygen limitation of *E. coli* JM109 in unbuffered LB-glycerol and TB medium. (A, B) Respiratory activity, (C, D) optical density, (E, F) pH, and (G−J) substrate and metabolite concentrations are dependent on oxygen supply. Unlimited oxygen supply: 400 rpm, 42% oxygen, and filling volume of (□) 10 or (◇) 20 mL. Limited oxygen supply: 200 rpm, 21% oxygen (air), and filling volume of (●) 10 or (▲) 20 mL.

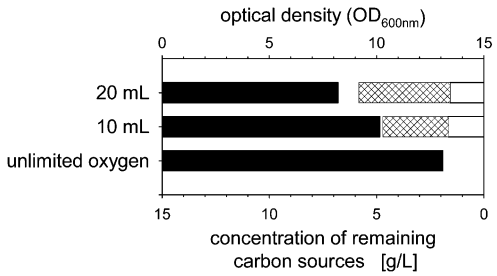

Figure 4. Summary of products from unbuffered LB-glycerol cultures in stationary phase (measured after 16 h), dependent on oxygen supply; 20 mL cultures were limited at 0.025 mol/L/h, and 10 mL cultures were limited at 0.045 mol/L/h: (black bars on left) cell density; (crosshatched bars) remaining glycerol; (open bars) acetate.

Production of BFD in *E. coli* SG13009. The cultivation in TB medium under unlimited oxygen supply was used to overexpress a mutant of benzoylformate decarboxylase (BFD) in *E. coli* SG13009. A LB culture was used for comparison. The growth rate of this strain is lower than that of JM109, leading to a lower rate of respiratory activity (Figure 5A). Application of oxygen-enriched air was not necessary to ensure unlimited oxygen supply. The final optical density of the culture under oxygen-unlimited condition (400 rpm, filling volume 10 mL) was 10-fold lower in LB medium as compared to that in TB medium (Figure 5B). Under oxygen-unlimited condition, the specific amount of BFD accumulated in the cytoplasm of cells grown in LB and TB media to the same extent (approximately 15% of total soluble protein), as calculated by the specific enzyme activity in cell extracts. Thus, volumetric productivity is proportional to cell density, resulting in a 10-fold increase of BFD production (approximately 0.5 g BFD per liter of culture liquid). Under oxygen-limited conditions (200 rpm, filling volume 15 mL or 150 rpm, filling volume 25 mL) using TB medium, the intracellular BFD concentration was increased, but fermentation time was extended (Figure 5A) and cell density was reduced (Figure 5B), especially in the culture with the lowest oxygen transfer capacity. Since the cell density was not affected by oxygen limitation in *E. coli* JM110 (Figure 3D), this effect is probably due to an inhibitory effect of the BFD on cell growth. We have shown that the amount of thiamine is limited in TB medium used for BFD overexpressing *E. coli* cells (unpublished results). The reason is that a large amount of thiamine is bound to BFD as a cofactor. Thiamine is also a cofactor of some enzymes in the central carbon metabolism of *E. coli*, thus leading to a competition between growth and product formation. It can be assumed that this competition is influenced in favor of the

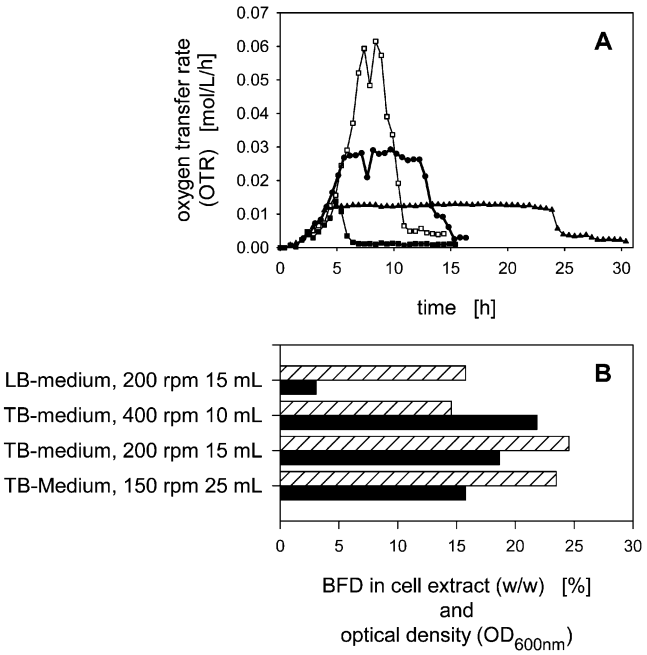

Figure 5. (A) Respiratory activity of *E. coli* SG13009 expressing benzoylformate decarboxylase (BFD) from *Pseudomonas putida*: (■) LB medium, 200 rpm, filling volume 15 mL, lead to unlimited oxygen supply; (□) TB medium, unlimited oxygen supply, 400 rpm, 21% oxygen (air), and filling volume of 10 mL; (●) TB medium at 200 rpm, filling volume 15 mL; (▲) TB medium at 150 rpm, filling volume 25 mL. Cultures were induced with 1 mmol/L IPTG at 3.5 h. (B) Products of *E. coli* SG13009 fermentation in LB medium and TB medium at different levels of oxygen supply: (hatched bars) proportion of BFD in cell extract; (black bars) cell density.

BFD formation, if growth is limited by insufficient oxygen supply. Oxygen limitation has been found to influence the expression of heterologous proteins in other cases; both increases and decreases have been reported (*25*). We suggest that oxygen supply should be considered as an important parameter for optimizing expression of individual proteins in small-scale cultures. This can easily be achieved by using different culture volumes. Once the optimal level of oxygen supply has been determined, the total amount of protein per culture flask can easily be raised with the following method: The culture volume per flask can be increased without changing the oxygen supply by simultaneously increasing the shaking speed proportionally. This procedure keeps the maximum oxygen transfer rate approximately constant, because oxygen supply is directly proportional to shaking speed and reversely proportional to the culture volume (*3*). In case larger amounts of proteins are required, the optimal oxygen transfer rate measured by the RAMOS device can also be used for scaling up to a fermentor.

Conclusions

Measuring respiratory activities of *E. coli* in different media using RAMOS made it possible to follow metabolic activity with a high time resolution and is therefore a suitable technique for optimizing culture conditions. Although the use of LB medium is prevalent in publications using small-scale cultures, significantly higher cell densities can be obtained with TB medium. Likewise, production of heterologous proteins can be enhanced. In situations where pH rises above 8 pose a problem, LB-glycerol at oxygen-unlimited conditions provides an alternative. Oxygen-unlimited conditions must be maintained when using LB-glycerol to prevent acidification. If possible, media containing glucose should generally be avoided for the cultivation of *E. coli* in shake flasks because this induces overflow metabolism, even under unlimited-oxygen conditions. During optimization of expression protocols using specific strains and constructs, the RAMOS device is an efficient tool to identify critical growth parameters. The OTR data presented here for JM110 can serve as a guideline for analyzing typical limitations in small-scale cultures, such as pH or product inhibition, oxygen limitation, or an unbalanced medium composition. The OTR curves measured for the optimization of BFD expression in *E. coli* SG13009 are qualitatively very similar to those for JM110 under the same growth conditions. This was also experienced for other strains (data not shown). Thus, optimized growth conditions for one *E. coli* strain can be transferred to other cultures. This can be useful when different strains of *E. coli* are used for cloning, screening, and production. However, the maximum of the OTR that can be reached by different strains, as well as their growth rate, may slightly vary. Therefore, different culture times have to be corrected for by choosing a harvest time at a comparable condition of the culture. For determining this culture condition the results from the RAMOS device are highly valuable.

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Note Added after ASAP. Bettina Frölich's name was misspelled in the version posted May 14, 2004; the corrected version was posted May 27, 2004.

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