Predictive Tool for Recombinant Protein Production in *Escherichia coli* Shake-Flask Cultures Using an On-Line Monitoring System

Martin Kunze, Robert Huber
AVT.Biochemical Engineering, RWTH Aachen University, Aachen 52074, Germany

Claudia Gutjahr and Stefan Müllner
Protagen AG, Dortmund 44227, Germany

Jochen Büchs
AVT.Biochemical Engineering, RWTH Aachen University, Aachen 52074, Germany

DOI 10.1002/btpr.719
Published online October 14, 2011 in Wiley Online Library (wileyonlinelibrary.com).

As Escherichia coli (E. coli) is well defined with respect to its genome and metabolism, it is a favored host organism for recombinant protein production. However, many processes for recombinant protein production run under suboptimal conditions caused by wrong or incomplete information from an improper screening procedure, because appropriate on-line monitoring systems are still lacking. In this study, the oxygen transfer rate (OTR), determined on-line in shake flasks by applying a respiration activity monitoring system (RAMOS) device, was used to characterize the metabolic state of the recombinant organisms. Sixteen clones of E. coli SCS1 with foreign gene sequences, encoding for different target proteins, were cultivated in an autoinduction medium, containing glucose, lactose, and glycerol, to identify relationships between respiration activity and target protein production. All 16 clones showed a remarkably different respiration activity, biomass, and protein formation under induced conditions. However, the clones could be classified into three distinct types, and correlations could be made between OTR patterns and target protein production. For two of the three types, a decrease of the target protein was observed, after the optimal harvest time had passed. The acquired knowledge was used to modify the autoinduction medium to increase the product yield. Additional 1.5 g/L glucose accelerated the production process for one clone, shifting the time point of the maximal product yield from 24 to 17 h. For another clone, lactose addition led to higher volumetric product yields, in fact 25 and 38% more recombinant protein for 2 and 6 g/L additional lactose, respectively. © 2011 American Institute of Chemical Engineers *Biotechnol. Prog.,* 28: 103–113, 2012
Keywords: E. coli screening, recombinant protein, autoinduction, oxygen transfer rate, respiration activity monitoring system on-line monitoring

Introduction

Recombinant DNA technology is a common tool for producing many different types of recombinant proteins such as enzymes, hormones, and cytokines. Many of these recombinant proteins are produced using genetically modified *Escherichia coli* (*E. coli*), because this bacterium is well known, allows easy introduction of foreign genes, grows fast on inexpensive media, and requires no complicated fermentation equipment. The economical feasibility of heterogeneous protein production processes depends on two things: acquiring high cell densities and high concentrations of target protein per cell. Unfortunately, these two requirements are not always given. In fact, there are several studies about how recombinant proteins actually affect the host organism, whereby these proteins may inhibit cell growth.[1] To avoid this, controllable expression systems have been developed[2] to separate cell growth and protein formation in two consecutive phases. After high biomass, concentrations are obtained in a fermenter, the foreign gene can be subsequently induced by environmental factors such as temperature,[3] dissolved oxygen tension,[4] and particular medium components.[5] An easy and comfortable approach to induce protein production at the proper time is to apply autoinduction media.[6–8] The autoinduction method works as follows: first, the production of the target protein is repressed by the primary carbon source (often glucose) of the organism. This repression initially guarantees undisturbed microbial growth, yielding high cell densities. Once this repressing substrate is fully depleted, the target protein production begins.

Though effective tools such as inducible expression systems and autoinduction media exist, the production of recombinant proteins influences the host organism's

Martin Kunze and Robert Huber contributed equally to this work.
Current address of Robert Huber: Sandoz, Biochemiestrasse 10, 6250 Kundl, Austria.
Correspondence concerning this article should be addressed to J. Büchs at jochen.buechs@avt.rwth-aachen.de.

metabolism. For many heterologous proteins, their overproduction leads to a metabolic burden,[9–12] which is defined as the amount of resources withdrawn from the host's metabolism. Lee and Ramirez[13] assumed that an additional load to the host's metabolism affects the cell growth in different ways. They mentioned typical behaviors where Type 1 is not affected by the protein formation, for Type 2 the cell growth rate decreases after induction but recovers later on, and Type 3, where the cells cannot regenerate from the shock caused by the induction. However, detailed knowledge about this is still lacking. Phenomena, such as metabolic burden and its effects on final product yields are still poorly understood, because of the lack of appropriate monitoring systems. An on-line system is described for quantifying the metabolic burden in a 50-L bioreactor where the respiratory activity is determined via exhaust gas analysis and biomass formation via ammonia consumption for pH control.[12,14] Nonetheless, such fermentations require considerable equipment, chemicals, and time. Huber et al.[15] presented a system for monitoring the influence of recombinant protein production on microscale, using a technique for scattered light and fluorescence measurement in shaken microtiter plates developed by Samorski et al.[16] and Kensy et al.[17] Conventional microtiter plates, however, run under process conditions which are not always comparable to those for larger scales, in particular in terms of oxygen transfer. Furthermore, for a direct on-line measurement, only proteins with special properties (e.g., fluorescence) are suitable.

In this article, the recombinant protein production of *E. coli* was investigated in shake flasks. The oxygen transfer rate (OTR), as determined by the respiration activity monitoring system (RAMOS), developed by Anderlei et al.,[18,19] was used as the characteristic parameter for measuring the metabolic state of the bacteria. Sixteen clones of *E. coli* K12 SCS1 bearing gene sequences for different foreign model proteins were studied. They were cultivated in an autoinduction medium to investigate the influence of heterogeneous protein formation to the host's respiration behavior. Consequently, the generated results were used to modify the autoinduction medium to increase target protein yield.

Methods

Organism

For all experiments, the *E. coli* K12 strain SCS1 (Stratagene) was used. Its genotype is *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^+ m_K^-) *supE44 relA1*. Sixteen clones, cited as PR01-16, have been kindly provided by Protagen AG (Germany), randomly chosen from a hEx1 cDNA library developed by Büssow et al.[20] These clones express different human model proteins from fetal brain tissue. SCS1 cells contain the plasmid pSE111 with the $argU^+$ gene to supply a rare t-RNA for arginine that improves expression of some target genes, the $lacI^Q$ gene to supply an additional *lac* repressor to reduce basal expression, and a gene encoding for kanamycin resistance. The used expression plasmid pQE30NST (Qiagen, Germany, GenBank accession no. AF074376) harbors the sequence for the target protein controlled by a T5 promoter under the control of a pair of *lac* operators. According to the manufacturer's specification, the vector is classified as low-copy plasmid with a copy number of approximately 20–30 per cell. Target proteins are equipped with N-terminal His_6-tags for affinity purification or antibody recognition.[21] Additionally, pQE30NST encodes for resistance to ampicillin.

Media and solutions

For growth under noninducing conditions, terrific broth (TB) medium consisting of 12 g/L tryptone, 24 g/L yeast extract, 12.54 g/L K_2HPO_4, 2.31 g/L KH_2PO_4, and 5 g/L glycerol (all ingredients from Roth, Germany) dissolved in water was used. The pH value was 7.2 ± 0.2 without adjustment.

For experiments under induced conditions, the Overnight-Express™ Instant TB medium (OnEx, Novagen, Merck Bioscience, Germany) was applied. OnEx is a commercially available ready-made autoinduction medium. It consists of complex components similar to TB medium and the additional carbon sources glucose, lactose, and glycerol. Similar media are available from Invitrogen™ (Magic Media™, Germany) and ForMedium™ (AIM, UK). For preparing the standard medium 60 g of granulate and 12.6 g of glycerol were dissolved in water and filled up to 1 L without pH-adjustment. high performance liquid chromatography (HPLC) analysis of the medium indicated a glucose concentration of 0.5 g/L and a lactose concentration of 2 g/L. After autoclaving, 100 mg/L ampicillin and 50 mg/L kanamycin were added to each medium under sterile conditions.

The OnEx autoinduction medium works as follows: glucose is the preferred carbon source and represses expression, lactose acts as the inducer of the recombinant expression system, and glycerol is an additional energy source. The values from HPLC analysis are in agreement with data of Studier,[7] where the design of a similar autoinduction medium is described. The working principle of this medium is that glucose is preferentially used as carbon source. During this initial growth phase, no other carbon source is metabolized. During this time, the production of the recombinant proteins is repressed as a result of catabolite repression of alternative carbon utilization pathways[22] and binding interactions between *lac* repressors and *lac* operators.[23] This allows an undisturbed growth of the microorganisms to high cell densities in the early stages of the cultivation. The depletion of glucose leads to a shift toward lactose import, and therefore, to the initiation of the recombinant protein expression.

Cultivation

For all cultivations, shake flasks with a nominal volume of 250 mL and equipped with a cotton plug were filled with 10 mL of medium. Cultures were grown at 37°C using an orbital shaker with a shaking diameter of 50 mm at a shaking frequency of 350 rpm. For precultivation, 10 mL of TB medium in shake flask was inoculated with 20 μL from a cryoculture, and cultures were grown for 8–10 h.

Experiments with OTR determination were performed with the in-house built RAMOS in modified shake flasks. Commercial versions are available from Kühner AG (Switzerland) and Hitec Zhang (Germany). To acquire samples for offline analysis, conventional shake flasks were used in parallel. To start the cultivation, the medium was inoculated from a preculture to an initial optical density (OD_{600}) of 0.1.

Analysis

OTR. The OTR during the cultivations was measured using the RAMOS. This device analyzes the oxygen

concentration in the gas headspace of the shake flask with an oxygen sensor.[18,19] For the measurement, the system uses a continually repeated cycle consisting of a rinsing and a measuring phase. During the rinsing phase (25 min), a controlled air flow is led through the shake flasks to adjust the equilibrium of the gas phase. The air flow is adjusted in such a way that the gas composition in the headspace of the RAMOS flasks is equal to that in conventional shake flasks equipped with a cotton plug.[24] Subsequently, the inlet and outlet valves, which are connected to the modified shake flasks, are closed to start the measuring phase (5 min). In the sealed system, the depletion of oxygen can be measured by the oxygen sensor on the top of each flask. The slope of this depletion corresponds to the OTR.

Biomass. To follow microbial growth, OD_{600} was determined from samples with a spectrophotometer at a wavelength of 600 nm (Uvikon 922A, Kontron Instruments, Italy) in 1.5-mL PS semimicrocuvettes (Plastibrand®, Brand GmbH, Germany) with a filling volume of 1 mL. Samples were diluted to keep the OD_{600} in the linear range from 0.05 to 0.5. Fresh medium was used for dilution and as a blank.

Substrates and Metabolites. The concentrations of the carbon sources glucose, lactose, and glycerol were determined by HPLC (Ultimate 3000, Dionex), equipped with a Carbohydrate PB2+ column (300 × 8 mm^2, CS Chromatographie Service, Germany). The column was eluted with filtered and degassed water at a flow rate of 0.6 mL/min and a temperature of 85°C. A detector with refractive index measurement was used (Shodex RH1, Showa Denko Europe GmbH, Germany).

Acetate was measured by HPLC (Dionex, Germany) equipped with a Organic Acid-Resin column (250 × 8 mm^2, CS Chromatographie Service, Germany). The eluent was 5 mM H_3PO_4 at a flow rate of 0.6 mL/min and 60°C. Peaks were detected by recording the refractive index (Skodex RI-71, Showa Denko Europe, Germany) or UV-absorbance at 220 nm with a diode array detector (UVD340S, Dionex, Germany). In both cases, the sample volume was 20 μL. For data analysis, the software Chromeleon (Dionex, Germany) was applied.

Recombinant Protein. The recombinant proteins were determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). After measuring the OD_{600} of the culture, samples were centrifuged at 14,000 rpm for 10 min. Subsequently, the supernatant was removed, and the pellet was resuspended with water to an OD_{600} of 25. To the 40 μL of this suspension, 140 μL of twofold concentrated sample buffer (E-PAGE Loading Buffer, Invitrogen, Germany) and 20 μL of dithiothreitol were added before shaking the mixture for 10 min at 1,000 rpm and 70°C in a thermo shaker. For analysis, the SDS-PAGE device (Invitrogen, Germany) was equipped simultaneously with up to two gels (4–12% Bis-Tris, Invitrogen, Germany). The transferred volumes were 20 μL for the prepared samples and 15 μL for the protein marker (Roti®-Mark Standard, Roth, Germany). The process was operated under the suggested standard settings of the manufacturer (running time 35 min, maximum current 200 V, and maximum power 0.25 W). The gels were stained in Simply Blue Staining solution (Invitrogen, Germany) at 37°C and shaken at 60 rpm overnight and were subsequently washed in water at the same temperature and shaking conditions for 2 h.

For quantitative analysis of the SDS gels by densitometry, one-dimensional gel analysis within the software TotalLab TL100 (Nonlinear Dynamics, UK) was used under the following conditions: (i) lane creation was done automatically; (ii) background substraction using rolling ball method with a radius of 100; and (iii) band detection using a minimum slope of 100. The resulting percentage of the target protein's band quantifies the recombinant protein fraction as part of the entire cell proteins (P_{rec}/P_{tot}, %). Assuming that biomass consists of 50% proteins, the overall volumetric product yield (Y_P, a.u.) of the cultivation can be estimated by multiplying P_{rec}/P_{tot} and 50% of biomass (OD_{600}, a.u.), as it can be seen from Eq. 1.

$$Y_P = P_{rec}/P_{tot} * 0.5 * OD_{600} \quad (1)$$

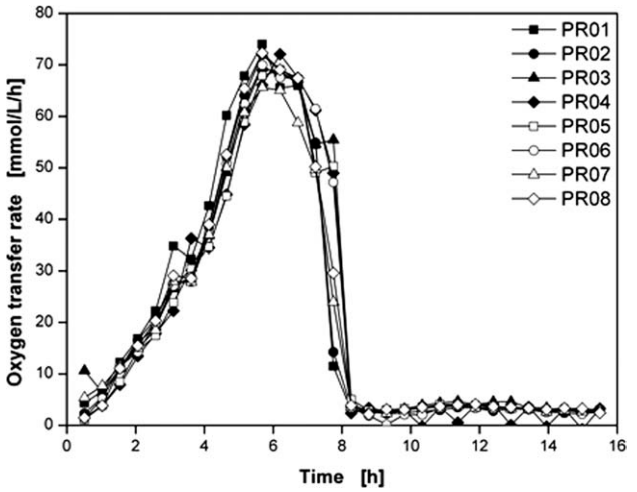

Figure 1. Cultivation of *E. coli* under noninduced conditions.

OTR during the cultivation of eight clones of *E. coli* SCS1 in noninducing TB medium (conditions: 250-mL flask, filling volume 10 mL, shaking frequency 350 rpm, shaking diameter 50 mm, and 37°C).

Results and Discussion

Cultivation under noninduced conditions

To cultivate the *E. coli* clones without inducing protein formation, the complex TB medium was applied, whereby glycerol served as the main carbon source. In the first experiment, eight different clones of *E. coli* SCS1 (PR01-08) were cultivated in the RAMOS device that measures online the respiratory activity. As shown in Figure 1, the OTR curves of all the clones depicted a very similar respiration behavior having an exponential increase with a maximum OTR of 65–75 mmol/L/h at 6 h. Afterward, there was a sharp drop in the OTR to nearly 0 mmol/L/h indicating that all of the original carbon sources in the medium were exhausted, and no further respiration could be observed until the end of the experiment. For the clones PR09-16, the same growth behavior was observed in an additional experiment (data not shown). SDS-PAGE analysis showed that no significant amounts of target proteins for all 16 clones were produced (data not shown).

The OTR curves in Figure 1 illustrate the typical growth of *E. coli* in TB medium as described earlier by Losen et al.[25] The maximum OTRs of 65–75 mmol/L/h observed here are in good agreement with results from other works, where *E. coli* was cultivated under similar conditions.

Palmen et al.[26] reached a value of 60 mmol/L/h in the same medium with a slightly lower shaking frequency of 320 rpm. A value of 65 mmol/L/h for a cultivation in a synthetic medium was also reported by Scheidle et al.[27] There are no indications for limiting or inhibitory factors. Although these *E. coli* clones bear plasmids with different gene sequences for recombinant proteins, these variations apparently have no influence on the organism's respiration under noninducing conditions. This fact indicates that combining the applied host organism with this vector system results either in no or only very low basal expression. The unintended induction, as it was described for some complex media by Grossman et al.,[28] was not observed in TB medium used during these experiments. Also, inhibitory effects caused by plasmid replication were not observed. Therefore, the low copy number of the applied vector system might be positive, as it was reported before that increased copy numbers can lead to decreased growth rates.[29,30]

Cultivation in autoinduction medium

To cultivate *E. coli* SCS1 under induced conditions, 16 clones were cultivated in the OvernightExpress™ TB autoinduction medium (OnEx). Though OnEx and TB medium are both complex media with glycerol as the major carbon source, it must be considered that there are significant differences in their compositions, resulting in different OTR curves. On the one hand, the glycerol concentration in TB medium is much lower, on the other hand OnEx contains the additional carbon sources glucose and lactose. Figure 2A shows the OTR curves during the cultivation of *E. coli* SCS1 in the inducing OnEx medium. Unlike the similar respiration patterns under noninduced conditions (Figure 1), the OTR curves in Figure 2A indicate a great difference in respiration under induced conditions. The variation in the metabolic activity of *E. coli* indicates that the DNA sequence encoding the target protein and varying from clone to clone indeed affect the host cells in quite different ways. The clones showing different OTR patterns can be classified into three distinct types:

- *Type 1* (*Figure 2A, white background, E. coli clones PR08, 11, 15, and 16*): This type showed an exponential increase in OTR from the beginning up to the time point when the maximum oxygen transfer capacity OTR_{max} of the culture system under the applied operating conditions of 67 mmol/L/h was reached, indicated by a shift to a horizontal plateau. The increase was only shortly interrupted at a time of 4–5 h. After 11–12 h, the initial carbon sources were depleted, so that the OTR dropped sharply. The subsequent peak was caused by the metabolism on acetate which was produced and accumulated in the earlier stages of the cultivation, as it is shown in following experiments (Figure 3A).
- *Type 2* (*Figure 2A, light-gray back ground, E. coli clones PR01, 02, 04, 05, 06, 07, 10, 12, 13, and 14*): For these clones, the first exponential increase of OTR was followed by a phase of more or less constant or even slightly decreasing OTR. This course apparently resulted from the recombinant protein being produced that led to a strong metabolic burden for the organisms, thereby inhibiting their respiration. The duration of this phase differed from clone to clone and ranged from 6 to 12 h. Subsequently, another exponential respiration phase occurred until a maximum OTR of 67 mmol/L/h was observed. At this level, there was either no or only a short period of oxygen limitation. This implied that the cells could regenerate themselves and continue to grow well. As the final peak indicates, acetate was also formed during the cultivation and metabolized after the depletion of glucose, lactose, and glycerol, as explained in more detail in Figure 3A.
- *Type 3* (*Figure 2A, gray background, E. coli clones PR03, 09*): After the first exponential respiration phase, these clones showed a continuous decrease in OTR under the applied experimental conditions, and no further increase was detected until the end of the fermentation. In this case, the negative effect of the recombinant protein formation was so strong that the cells could not recover.

To quantify the recombinant proteins, the cells were analyzed by SDS-PAGE (Figure 2B). Only nine of the 16 clones depicted clear product bands (PR01, 02, 03, 04, 05, 09, 10, 11, and 13). It was yet unclear why some of the clones (PR06, 07, 08, 12, 14, 15, and 16) did not show recombinant protein, though their OTR curves indicated a deviation from uninduced conditions. One reason might be that the synthesized product was subsequently degraded by proteolysis, so that no protein remained after 24 h of cultivation. Another explanation might be that the DNA sequence for the target protein was only transcribed to mRNA, and *E. coli* was not able to translate it to the protein. In this case, the bacterial growth could have been inhibited by the overloaded transcription system. It is important to consider that Figure 2B is only a snapshot at the end of the cultivation, as it is routinely performed in many laboratories. Therefore, this conventional endpoint determination may not give a complete picture about what actually happens during the cultivation.

For further characterizing the aforementioned clone types, one clone from each group was chosen for an additional RAMOS experiment with parallel offline analysis of biomass (OD_{600}), sugar consumption, acetate production, and pH-value from conventional shake flasks (Figure 3A). It is obvious that clone PR15, representing Type 1, started with an exponential respiration phase where only glucose was consumed. After glucose was depleted, lactose and glycerol were consumed in parallel. This fact does not agree with an earlier report where lactose and glycerol are consumed one after another.[31] After 7 h, the OTR reached its maximum, and the following oxygen limitation caused a change to linear biomass formation. The insufficient oxygen supply also led to an increase in the acetate concentration to a maximum of 4.5 g/L and a minimal pH-value of 6.5 after 12 h. The strong decrease in the pH-value indicates that the buffer capacity of the medium was depleted. After 12 h, all initially added carbon sources were exhausted, and the OTR dropped. As mentioned before, the subsequent OTR peak was caused by acetate utilization leading to further respiration activity. As a consequence of acetate consumption and ammonia release from amino acids in the complex medium being metabolized, pH increased to a higher value of 7.6. After 16 h, no further change in the concentrations was observed.

PR05, representing Type 2, showed a shorter exponential respiration phase in the beginning of the cultivation. Because of the depletion of glucose and the induction by lactose, the OTR and cell growth rate slightly decreased. Contrary to Type 1 (PR15), the consumption of lactose and glycerol was slower. After 9 h, all of the lactose was consumed. Thus, unaffected cell growth was again possible resulting in an exponential increase in OTR and OD_{600}. Moreover, after 14 h the residual glycerol in the medium was depleted. PR05 produced considerably less acetate during cultivation than

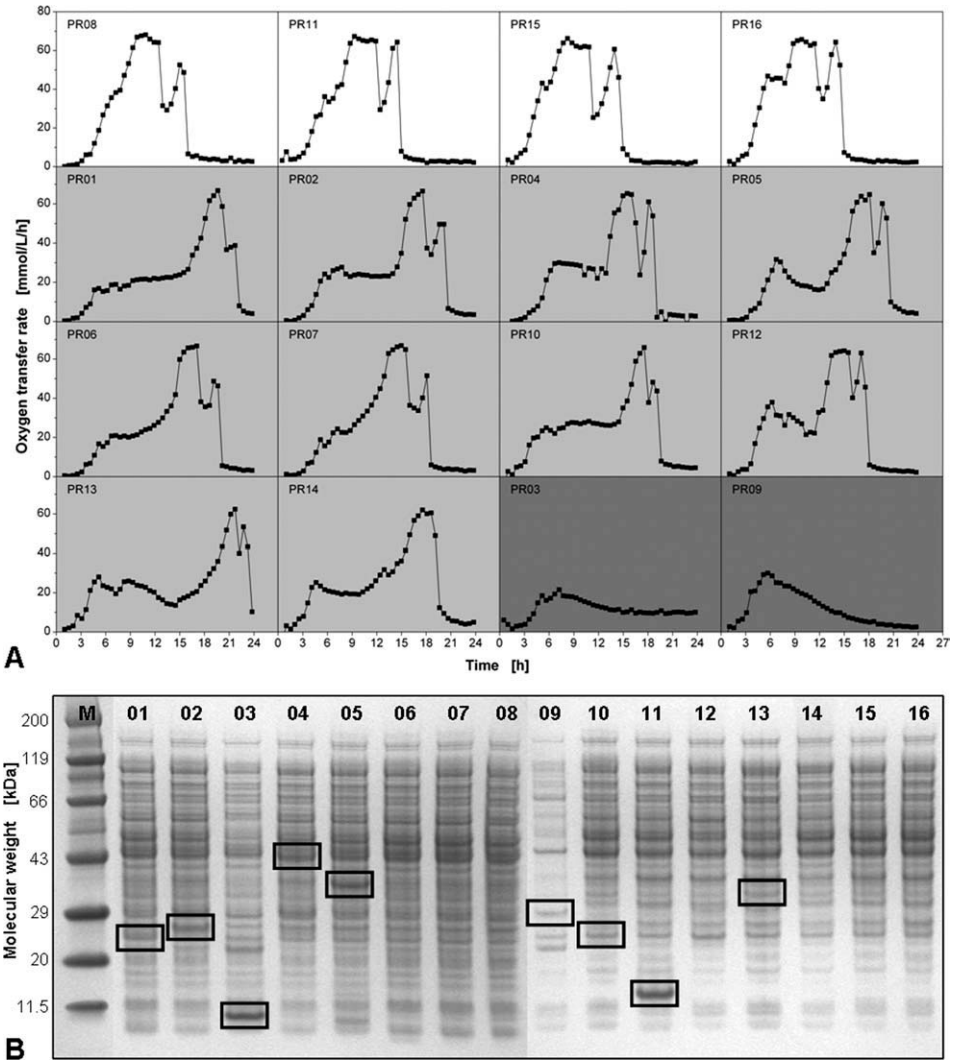

Figure 2. Cultivation of *E. coli* under induced conditions using an autoinduction medium.

OTR during the cultivation (A) and SDS-PAGE analysis showing protein per biomass after 24-h cultivation (B, target protein framed, M = protein marker) of 16 clones of *E. coli* SCS1 expressing different recombinant target proteins in OvernightExpress™ autoinduction medium with three different respiration behaviors observed: Type 1 (white background); Type 2 (light-gray background); and Type 3 (dark-gray background; conditions: 250-mL flask, filling volume 10 mL, shaking frequency 350 rpm, shaking diameter 50 mm, and 37°C).

PR15, because there was no extensive oxygen limitation. In addition, the pH did not decrease because of the lower acetate formation and the sufficient buffer capacity in the medium for these experimental conditions.

PR03, representing Type-3 clones, depicted initial exponential increase of OTR on glucose, followed by a phase of constant OTR and weak cell growth until the end of the cultivation after 22 h. Compared to PR05 and 15, lactose and glycerol were consumed much slower. After 16 h, no more lactose was found in the medium, and cell growth did not resume, as it was observed for Type-2 clones, though glycerol was not depleted. Acetate was formed during the entire cultivation. This must have been caused by the overflow metabolism, because no oxygen limitation was present under the applied process conditions.

Figure 3B shows the formation of the recombinant proteins during the cultivation for PR15, 05, and 03. It can be seen that the protein formation behavior of the three types differed tremendously. They all have in common that no target protein was formed during the first 3 h of cultivation. This shows the effect of the autoinduction medium where glucose in the medium initially repressed protein production to avoid an interference with cell growth in the beginning of the cultivation. After 5 h of cultivation, the respective proteins were found for all three clone types. It can be seen that Type 3 (PR03) was continuously producing its protein over the whole process time. The analysis by densitometry showed a constant high level of 21–25% for the target protein fraction (Figure 3A; P_{rec}/P_{tot}) after 8-h cultivation. This correlates very well with the slow consumption of lactose and inhibited cell growth. PR05, representing Type 2, showed the maximum quantity of recombinant protein per cell after 8 h. The corresponding value for P_{rec}/P_{tot} was 16%. At this time, lactose was depleted, and the second exponential growth phase started with the residual glycerol as the sole carbon source. Therefore, the following decrease in the protein content per cell was caused by an increase in noninduced biomass and a "dilution" of the target protein in the cells. After 16 h, when no carbon source was left in the medium and the growth stopped, the protein concentration remained constant at a much lower level resulting in a P_{rec}/P_{tot} level of 10%. PR15, representing Type-1 clones, was

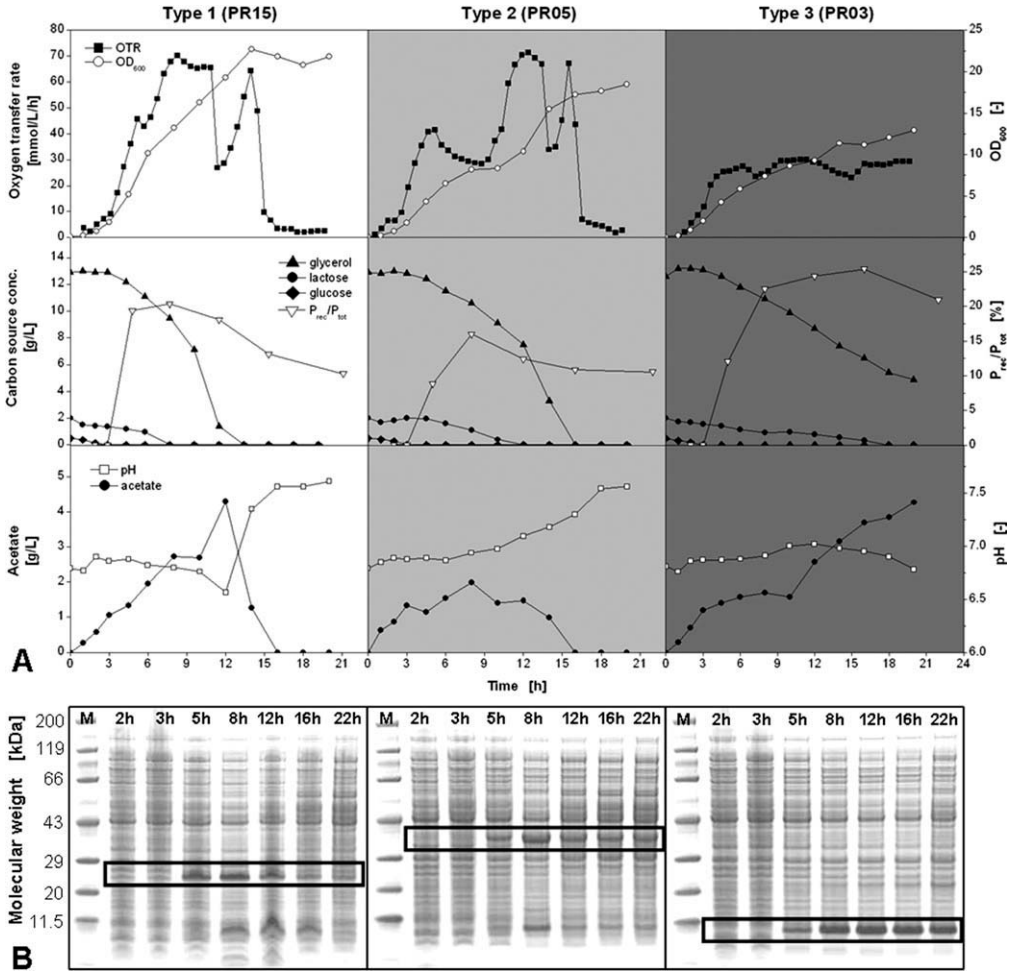

Figure 3. Further characterization of three *E. coli* clones with different respiration behaviors under induced conditions.

Characteristic growth parameters (A) and SDS-PAGE analysis showing protein per biomass after 2-, 3-, 5-, 8-, 12-, 16-, and 22-h cultivation (B, target protein framed, M = protein marker) of three clones of *E. coli* SCS1 with different respiration behaviors during the cultivation in OvernightExpress™ autoinduction medium. Type 1 (white background); Type 2 (light-gray background); and Type 3 (dark-gray background; conditions: 250-mL flask, filling volume 10 mL, shaking frequency 350 rpm, shaking diameter 50 mm, and 37°C).

able to form its target protein in the time range from 5 to 12 h, as shown in Figure 3B. In this time, the percentage of the target protein reached a maximal value of 20%. However, after 8 h another protein band of lower molecular weight was found. At the end of the cultivation, both these proteins disappeared. Presumably, the recombinant protein was the target of proteolytic degradation where the aforementioned smaller protein was the first degradation product. This effect was observed for another Type-1 clone, too, as PR16 showed good productivity until 16 h of cultivation but no target protein at all after 24 h (data not shown). For clone PR03 and PR05, no such degradation was found, as PR03 showed a constant high productivity until the end of the cultivation, and PR05 had a constant product level per cell over the last 6 h. This demonstrates that some of the proteins were more prone to proteases than others, thereby verifying the assumption of proteolytic degradation mentioned before. It is, therefore, extremely important to characterize the end of the production for each individual clone or strain by on-line monitoring and to take the sample at this time point for product quantification.

It is known that cells containing expression plasmids that confer resistance to ampicillin can readily be overgrown by cells that have lost the plasmid, particularly if the target protein is stressful to the host. The enzyme β-lactamase can be secreted to the medium where it inactivates all of the ampicillin by the time a culture has become visibly turbid.[32] Thus, cells that have lost plasmid can overgrow cells that are capable of producing target protein, and therefore, leading to an increasing OTR but decreasing product yield. Such overgrowth may well be responsible for the second increase in the OTR patterns of Type-2 clones. Nevertheless, the following results (Figures 6 and 7) indicate that the second increase of the OTR is not due to overgrowth of the cultures by cells that have lost plasmid.

As mentioned in "Introduction" section, there are several publications about the influence of recombinant protein formation on the metabolism of the expression host. Similar to the three clone types described earlier, also Lee and Ramirez[13] reported on such a different behavior with three distinct types, where the additional load to the host's metabolism affects the cell growth in different ways. Their hypothesis agrees very well with the phenomena observed in this study. However, the reason for this different behavior is yet unclear. Büssow et al.[33] already reported that for the applied cDNA-library, from which our clones were selected, no significant correlation was found between the expression results and either the protein size or the mean net charge. Further investigations within this work excluded that the protein size, 10–55 kDa in our case, the isoelectric point, 6.45–

11.35, the hydrophobicity, indicated by the grand average of hydropathy value and reaching from −0.899 to −0.003, and the aliphatic index, which indicates for the fraction of aliphatic amino acids in the proteins and reaches from 37.06 to 97.66 for our proteins, are responsible for the observed phenomena. All parameters were determined via the ExPASy ProtParam tool (ExPASY, Switzerland, data not shown). An explanation for the different expression types might be the different codon usages of *E. coli* proteins and the target proteins. Resulting insufficiencies of the tRNA pool can lead to differences in microbial growth and recombinant protein formation.[34]

Using conventional screening protocols, especially in microtiter plates and shake flasks, the cultivation and product formation are evaluated via few data points, originating mostly from endpoint analysis. The data presented here show that the results at the end of the experiment often do not reflect the real course of the process. In contrast, the on-line OTR signal from RAMOS gives important information over the whole fermentation time. Interestingly, for PR05 and PR03 representing clones Types 2 and 3, respectively, a direct relationship of the product formation and the OTR patterns could be observed. Whereas PR03 shows a constant inhibited respiration activity due to slow continuous protein production, the OTR curve of PR05 indicates the optimal point for terminating the process and harvesting the cells to have the maximum product yield without loss of time and resources. Consequently, RAMOS is a good tool to select and characterize most favorable clones with respect to biomass and target protein production. Additionally, it enables a rough prediction of the expression result without performing costly and laborious offline analysis.

Modification of the autoinduction medium

As the applied autoinduction medium OnEx is a commercially available product, it was developed to suit a great variety of applications. Nonetheless, Blommel et al.[31] already reported that due to the multiple carbon sources present in the medium, their relative amounts critically contribute to the outcome of the autoinduced process. This was also confirmed in this article. In particular, clone PR05, representing clone Type 2, is a good example for the suboptimal medium composition. In addition, also the product formation with clone PR03, representing Type 3, shows potential for improvement. Consequently, the OnEx autoinduction medium was modified to optimize the production process and to validate the concept of using RAMOS as a screening tool for recombinant protein production.

Glucose Concentration. The first aim was to accelerate the product formation in Type-3 clone PR03 to increase the space-time yield. By applying the approach of higher glucose concentrations, the time of induction was shifted to a later point to reach higher cell densities before the target protein production started. As reported before, the time of induction can strongly influence the result of cell growth and protein formation.[5,15,35] Increasing the glucose concentration of the autoinduction medium should lead to more inducible cells, and hence, to a higher product yield in an earlier stage of the process. This makes, of course, only sense, if culture conditions are selected which provide high levels of maximum oxygen transfer capacity. Otherwise a higher biomass concentration only results in a stronger oxygen limitation. With conventional OnEx, the glucose concentration amounted to 0.5 g/L. This medium was supplemented with additional glucose, resulting in final concentrations of 1–5 g/L. In Figure 4A, the OTR curves for the respective cultivations are depicted. The unmodified medium showed the typical curve for Type-3 clones with an exponential increase in the beginning and subsequent slightly decreasing OTR until the end of the cultivation. With 0.5, 1.5, and 4.5 g/L additional glucose, the OTR level after the first exponential phase increased from 21 to 28, 32, and 49 mmol/L/h, respectively, due to increased biomass before the induction by lactose set in. Surprisingly, the OTR patterns for additional 0.5 and 1.5 g/L glucose looked similar to those of Type-2 clones (Figure 2) with a constant level until 18 h followed by another exponential increase. With 4.5 g/L more glucose, there was a further change of the OTR profile, comparable to that of Type 1, with only a short interruption of the exponential growth and a long phase of oxygen limitation. The OD_{600} in nonmodified OnEx medium was 9.8 after 10 h of cultivation (Figure 4B). The value for the medium with the additional 0.5, 1.5, and 4.5 g/L glucose rose to 11.0, 13.8, and 15.7, respectively. In a later phase, the biomass in the conventional medium increased with a constant growth rate, whereas for the medium variants with additional 0.5 and 1.5 g/L glucose, a higher growth rate after 17 h was observed when the OTR increased again. With 4.5 g/L more glucose, this effect occurred even earlier, indicated by a strong increase of OD_{600} in the time from 10 to 15 h.

Figure 4B and the SDS gels in Figure 4C show that the amount of recombinant protein per cell during cultivation was different for the tested medium variants. Nonmodified OnEx had a nearly constant product level over the whole time with P_{rec}/P_{tot} of 22–24%. In the media with 0.5 and 1.5 g/L more glucose, the biomass specific product yield decreased after 17 h from more than 20 to 12% at the end. This fact suits very well to the growth and expression behavior of Type-2 clones (Figure 3), where the growth rate again increased when the inducer lactose in the medium was depleted, causing the growth of noninduced cells and, therefore, leading to a "dilution" of the product inside the cells. Additional 4.5 g/L glucose resulted in a strong decrease of the product yield, so that after 15 h almost no recombinant protein was found. Apparently, the maximal level of the target protein occurred already within the first 10 h. So, the optimal harvest point for this medium variant would have been earlier. By considering the overall product yield (Eq. 1) it can be seen that conventional OnEx showed a maximum of 188 a.u. at the end of the cultivation. With 1.5 g/L additional glucose, the maximal value of 182 a.u. was already achieved after 17 h.

Altogether, it was proven that additional glucose in the medium led to the desired aim. In fact, higher biomass yields in the early stages of cultivation were obtained due to a longer undisturbed growth phase in the beginning. As a consequence, the time point of the maximal product yield, and therefore, the optimal harvest point were shifted to an earlier time. Furthermore, the results show a strong dependency of growth and expression behavior on the medium composition. By adding more glucose to the medium, *E. coli* PR03 changed from a Type-3 to a Type-2 clone, and it can be assumed that a further increase (more than 5 g/L) even might result in the change to Type 1.

Glycerol Concentration. Another modification of the autoinduction medium involved the cultivation without glycerol. In Figure 3B, it was shown for clone PR05 that glycerol in the medium caused the growth of noninduced cells

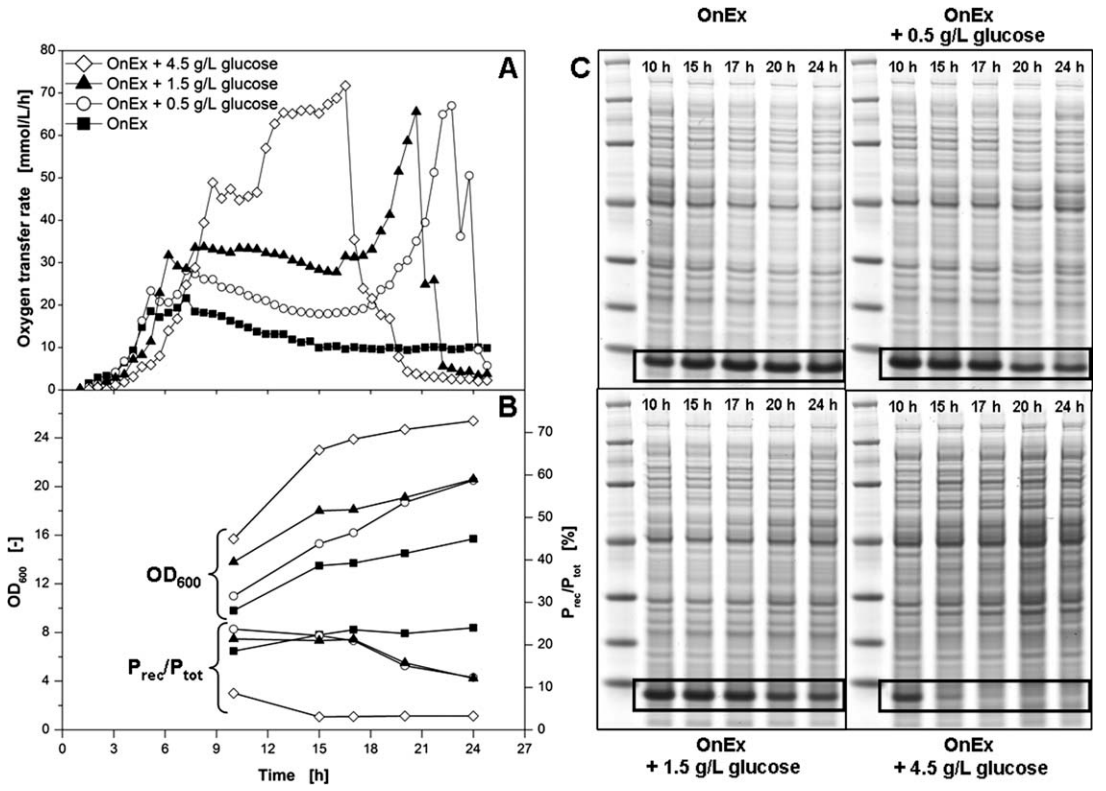

Figure 4. Cultivation of *E. coli* in autoinduction medium with various glucose concentrations.

OTR (A), OD_{600}, and recombinant protein fraction per biomass analyzed by densitometry (B) and SDS-PAGE analysis showing protein per biomass (C, target protein framed, M = protein marker) during the cultivation of *E. coli* SCS1 PR03 in OvernightExpress™ autoinduction medium with various glucose concentrations (conditions: 250-mL flask, filling volume 10 mL, shaking frequency 350 rpm, shaking diameter 50 mm, and 37°C).

after lactose was depleted and product formation subsided, thereby resulting in a smaller yield of recombinant protein per biomass. By omitting glycerol, this second growth phase should be avoided.

Figure 5A compares the cultivations with and without glycerol in the medium. For the glycerol containing medium, the typical OTR curve of clone Type 2 was obtained. The medium lacking glycerol showed a completely different respiration behavior without any indications of recombinant protein production. The respiration rather resembled that in noninducing TB medium (Figure 1). In this medium, there was a strong increase in the OTR to a maximum level of 65 mmol/L/h and a subsequent drop when all carbon sources in the medium were depleted. The SDS gel image and densitometric analysis in Figure 5B supports the assumption that there was no significant protein formation during the cultivation without glycerol. Whereas a strong product band was formed in the conventional OnEx medium (P_{rec}/P_{tot} = 7.8%), it was remarkably lower for the modified medium (P_{rec}/P_{tot}= 5.1%). Additionally, the biomass yield was much lower without glycerol, resulting in a strong decrease of the overall volumetric product yield (Eq. 1) from 82 a.u. for the nonmodified medium to 30 a.u. in the glycerol free variant. Apparently, glycerol acted as an additional substrate during the protein production, and therefore, decelerated the consumption of the inducer lactose. Without glycerol in the medium, lactose as the sole carbon source after glucose depletion was metabolized so quickly that the inducing function of lactose did not work properly. Thus, totally omitting glycerol cannot be recommended. Consequently, finding the right glycerol concentration for each particular clone is important to achieve the maximal target protein yield. The optimal glycerol concentration would be found, if there was no further increase in the OTR, representing the growth of noninduced cells, as it was obtained here after 9 h (Figure 5A, OnEx).

Lactose Concentration. Another optimization strategy for maximizing the product yield was to increase the lactose concentration to extend the target protein formation phase and avoid the growth of noninduced cells. An additional 2 and 6 g/L lactose was, respectively, supplemented to the conventional OnEx medium, so that the final concentration corresponded to twice and four-times that of the original concentration of 2 g/L. The RAMOS results are depicted in Figure 6A. The unmodified OnEx medium again showed the typical OTR curve of Type-2 clones. As it was intended, the increased lactose concentration caused a longer protein formation phase. Additional 2 g/L lactose led to an extension of this phase from 4 to 8 h. The additional 6 g/L lactose extended the production phase to 16 h with no subsequent growth of noninduced cells. Interestingly, the duration of the OTR plateau at around 25 mmol/L/h (time of protein formation) correlates very well with the lactose concentration. The double and fourfold amount of the inducer lactose resulted in production times that were two-times and four-times longer, respectively, as judged from the OTR curves. The SDS gel image and densitometric data in Figure 6B support these results, whereby stronger product bands were found with increasing lactose concentrations, and P_{rec}/P_{tot} increased from 7.8% with no added lactose to 10.3 and 12.1% with 2 and 6 g/L more lactose, respectively. Though more substrate was available to the cells, the final biomass (OD_{600}) slightly

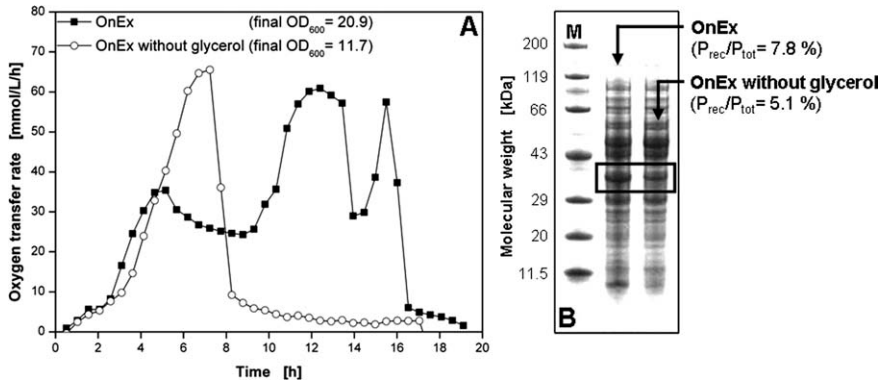

Figure 5. Cultivation of *E. coli* in autoinduction medium with various glycerol concentrations.

OTR during the cultivation (A) and SDS-PAGE analysis showing protein per biomass after 19 h of cultivation (B, target protein framed, M = protein marker) of *E. coli* SCS1 PR05 in OvernightExpress™ autoinduction medium with and without glycerol (conditions: 250-mL flask, filling volume 10 mL, shaking frequency 350 rpm, shaking diameter 50 mm, and 37°C).

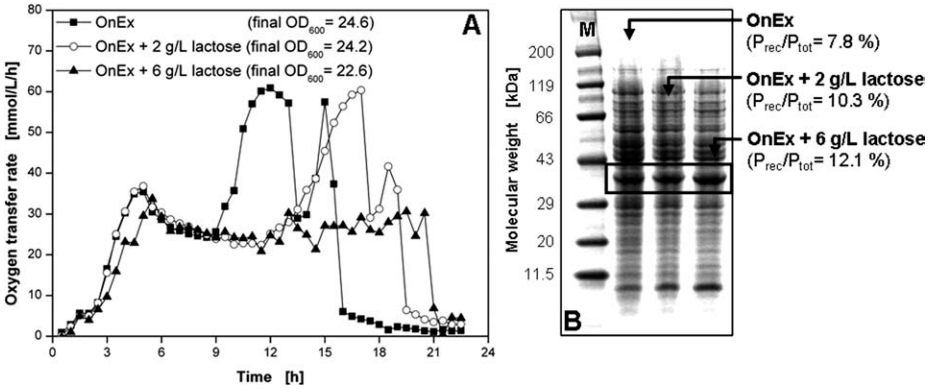

Figure 6. Cultivation of *E. coli* in autoinduction medium with various lactose concentrations.

OTR during the cultivation (A) and SDS-PAGE analysis showing protein per biomass after 18 h of cultivation (B, target protein framed, M = protein marker) of *E. coli* SCS1 PR05 in OvernightExpress™ autoinduction medium with various lactose concentrations (conditions: 250-mL flask, filling volume 10 mL, shaking frequency 350 rpm, shaking diameter 50 mm, and 37°C).

decreased with rising lactose concentration, indicating that more resources were withdrawn from growth to product formation. Taking all together, the overall volumetric product yield (Eq. 1) increased from 100 a.u. in OnEx to 125 and 138 a.u. in OnEx with 2 and 6 g/L additional lactose. In addition to the band of the target protein, another one of lower molecular weight (~10 kDa) occurred in the gel. The reason for that is yet unclear.

Figure 7 illustrates in more detail the different growth and target protein production behavior for conventional OnEx and OnEx containing 6 g/L more lactose. In addition to the OTR, Figure 7A also depicts the respective OD_{600} curves. It can be seen that the OD_{600} curves were identical to each other up to 8 h. Subsequently, the OD_{600} curve for unmodified OnEx medium increased stronger, thereby, indicating a higher growth rate due to the missing target protein formation after inducer depletion. The final OD_{600} value after 19 h for the unmodified medium is 10% higher than that for the modified one. However, comparing Figure 7B,C (see frames) shows that additional lactose strongly promoted recombinant protein formation. Without additional lactose, the protein amount per cell reached its maximum after 6–8 h with 12.4% of target protein. Thereafter, it decreased until it stayed constant after 14 h (P_{rec}/P_{tot} = 8.3%) when the OTR dropped, thus indicating that no carbon source was left in the medium. This clearly shows that the growth of noninduced cells is responsible for the lower product yield. With additional lactose, this phenomenon was avoided by extending the protein formation phase until 18 h (Figure 7C). After 6 h, the biomass specific product yield had already reached its maximum and remained nearly constant at 14% until the end of the cultivation after 19 h. It can be assumed that during protein production the glycerol from the medium was fully consumed, so that growth and protein production stopped at the same time point. This led to a constant high level of the desired protein in the cells until the end of the cultivation. As a further advantage, additional 6 g/L lactose in the medium prevented an oxygen limitation during the process, and therefore, the production of potentially inhibiting by-products.

The results presented in Figures 6 and 7 also prove that the overgrowth by cells having lost their plasmid is not responsible for the second increase of the OTR and the corresponding decrease of the biomass specific protein yield. As with additional lactose in the medium, a constant high product yield was observed until the end of the cultivation, the appearance of uninduced cells in the conventional autoinduction medium is more likely.

Consequently, adding lactose to the medium is a simple and inexpensive way to increase the product yield. For a further increase, a lactose fed-batch would be desirable. It was already shown that an appropriate feeding strategy can lead

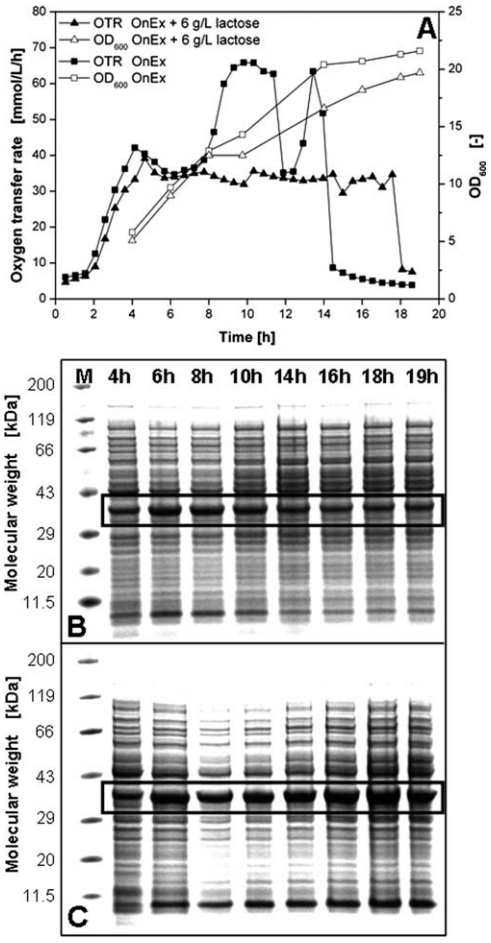

Figure 7. Further characterization of growth and product formation of *E. coli* in autoinduction medium with various lactose concentrations.

OTR and OD_{600} during the cultivation (A) and SDS-PAGE analysis showing protein per biomass after 4, 6, 8, 10, 14, 16, 18, and 19 h of cultivation (target protein framed, M = protein marker) of *E. coli* SCS1 PR05 in conventional OvernightExpress™ autoinduction medium (B) and with additional 6 g/L lactose (C; conditions: 250-mL flask, filling volume 10 mL, shaking frequency 350 rpm, shaking diameter 50 mm, and 37°C).

to, both, higher biomass and biomass specific product yield.[36] Taking the results of the glycerol experiments (Figure 5) into consideration, it is recommended to use both lactose and glycerol as carbon sources for the feeding solution. Otherwise, lactose would be consumed so fast that the induction would be ineffective.

Conclusion

It was found that among the 16 examined *E. coli* SCS1 clones there was a great variance with respect to respiration activity and target protein production, despite using the same recombinant expression system. However, it was possible to classify them into three distinct types. Correlations between OTR, indicating metabolic activity, and the recombinant protein formation could be established. Phases of lower OTR were identified as strong production phases, whereas exponential increases of the OTR indicated undisturbed cell growth with low productivity.

It must be considered that the results shown in this work are not yet focused on a detailed quantitative characterization of the relationship among OTR, microbial growth, and recombinant gene expression. For that purpose, an in-depth statistical analysis would be necessary. However, very similar qualitative differences in respiration and expression behavior were also found for other clone banks in our laboratory. In fact, this work gives a first mechanistic understanding of how respiration activity, biomass formation, and recombinant protein formation interact. It allows rough predictions about the productivity of a particular clone without costly offline analysis. Furthermore, this knowledge can be used to optimize process conditions. Several strategies have been demonstrated to increase the productivity by varying the medium composition, in particular the carbon sources glucose, glycerol, and lactose. Currently, investigations are performed to develop a tailor-made screening protocol for the three different expression types.

Unlike conventional shake-flask techniques in which only endpoint concentrations of biomass and product are measured, using the RAMOS device has the following advantages:

- It is fast, allowing relatively immediate measurement (e.g., every 30 min, depending on the settings of the device) of the relevant parameter OTR, characterizing the metabolic state of the microorganism.
- It is noninvasive: the cultures need not to be disturbed during cultivation.
- It reduces the need for sampling, as from the OTR curves relevant sampling points can be defined on-line.

This study also shows that unexpected phenomena such as the observed ones are hardly detectable using conventional screening protocols. This might lead to wrong assumptions about the selection of production strains, process parameters, and optimal point of harvest, ultimately, to wasted resources. As screening protocols differ from working group to working group,[37] the results found might give important hints for investigations in the field of recombinant protein expression with *E. coli*.

Up to now, the reason for the observed phenomena is yet unclear, as there was no correlation between the expression behavior and parameters such as protein size, mean net charge, isoelectric point, hydrophobicity, and aliphatic index. Therefore, future investigations should also focus on the recombinant protein properties and their influence to the microbial growth and expression behavior. Furthermore, it has not been investigated how plasmid stability and plasmid loss under the applied conditions affects the expression result. Because of the selective pressure in a microbial culture, cells may reduce their plasmid copy number or even lose the gene of interest within the plasmid; this would shift the metabolism from production to growth due to a lower metabolic burden, and consequently, lead to lower product yields. Thus, measuring plasmid contents, for example, by flow cytometry, could give further important information for a more detailed understanding of the respiration and expression behaviors observed in this study.

Acknowledgments

The financial support from the Stiftung Industrieforschung (Project S735) is gratefully acknowledged. This work was performed as part of the Cluster of Excellence 236 "Tailor-Made Fuels from Biomass", which is funded by the excellence initiative by the German federal and state governments to promote science and research at German universities. Further, the authors thank M.J. Blümich for proof-reading the manuscript.

Literature Cited

1. Bonomo J, Gill RT. Amino acid content of recombinant proteins influences the metabolic burden response. *Biotechnol Bioeng*. 2005;90:116–126.
2. Makrides SC. Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol Rev*. 1996;60:512–538.
3. Yabuta M, Onaimiura S, Ohsuye K. Thermo-inducible expression of a recombinant fusion protein by *Escherichia coli* lac repressor mutants. *J Biotechnol*. 1995;39:67–73.
4. Khosla C, Curtis JE, Bydalek P, Swartz JR, Bailey JE. Expression of recombinant proteins in *Escherichia coli* using an oxygen-responsive promoter. *Biotechnology*. 1990;8:554–558.
5. Neubauer P, Hofmann K, Holst O, Mattiasson B, Kruschke P. Maximizing the expression of a recombinant gene in *Escherichia coli* by manipulation of induction time using lactose as inducer. *Appl Microbiol Biotechnol*. 1992;36:739–744.
6. Sreenath HK, Bingman CA, Buchan BW, Seder KD, Burns BT, Geetha HV, Jeon WB, Vojtik FC, Aceti DJ, Frederick RO, Phillips GN, Fox BG. Protocols for production of selenomethionine-labeled proteins in 2-L polyethylene terephthalate bottles using auto-induction medium. *Protein Expr Purif*. 2005;40:256–267.
7. Studier FW. Protein production by auto-induction in high-density shaking cultures. *Protein Expr Purif*. 2005;41:207–234.
8. Tyler RC, Sreenath HK, Singh S, Aceti DJ, Binginan CA, Markley JL, Fox BG. Auto-induction medium for the production of [U-N-15]- and [U-C-13, U-N-15]-labeled proteins for NMR screening and structure determination. *Protein Expr Purif*. 2005;40:268–278.
9. Bentley WE, Mirjalili N, Andersen DC, Davis RH, Kompala DS. Plasmid-encoded protein: the principal factor in the metabolic burden associated with recombinant bacteria. *Biotechnol Bioeng*. 1990;35:668–681.
10. Bhattacharya SK, Dubey AK. Metabolic burden as reflected by maintenance coefficient of recombinant *Escherichia coli* overexpressing target gene. *Biotechnol Lett*. 1995;17:1155–1160.
11. Glick BR. Metabolic load and heterologous gene-expression. *Biotechnol Adv*. 1995;13:247–261.
12. Hoffmann F, Rinas U. On-line estimation of the metabolic burden resulting from the synthesis of plasmid-encoded and heat-shock proteins by monitoring respiratory energy generation. *Biotechnol Bioeng*. 2001;76:333–340.
13. Lee J, Ramirez WF. Mathematical-modeling of induced foreign protein-production by recombinant bacteria. *Biotechnol Bioeng*. 1992;39:635–646.
14. Schmidt M, Viaplana E, Hoffmann F, Marten S, Villaverde A, Rinas U. Secretion-dependent proteolysis of heterologous protein by recombinant *Escherichia coli* is connected to an increased activity of the energy-generating dissimilatory pathway. *Biotechnol Bioeng*. 1999;66:61–67.
15. Huber R, Ritter D, Hering T, Hillmer AK, Kensy F, Muller C, Wang L, Büchs J. Robo-Lector—a novel platform for automated high-throughput cultivations in microtiter plates with high information content. *Microb Cell Fact*. 2009;8:42.
16. Samorski M, Muller-Newen G, Büchs J. Quasi-continuous combined scattered light and fluorescence measurements: a novel measurement technique for shaken microtiter plates. *Biotechnol Bioeng*. 2005;92:61–68.
17. Kensy F, Engelbrecht C, Büchs J. Scale-up from microtiter plate to laboratory fermenter: evaluation by online monitoring techniques of growth and protein expression in *Escherichia coli* and *Hansenula polymorpha* fermentations. *Microb Cell Fact*. 2009;8:68.
18. Anderlei T, Büchs J. Device for sterile online measurement of the oxygen transfer rate in shaking flasks. *Biochem Eng J*. 2001;7:157–162.
19. Anderlei T, Zang W, Papaspyrou M, Büchs J. Online respiration activity measurement (OTR, CTR, RQ) in shake flasks. *Biochem Eng J*. 2004;17:187–194.
20. Büssow K, Cahill D, Nietfeld W, Bancroft D, Scherzinger E, Lehrach H, Walter G. A method for global protein expression and antibody screening on high-density filters of an arrayed cDNA library. *Nucleic Acids Res*. 1998;26:5007–5008.
21. Büssow K, Nordhoff E, Lubbert C, Lehrach H, Walter G. A human cDNA library for high-throughput protein expression screening. *Genomics*. 2000;65:1–8.
22. Hogema BM, Arents JC, Bader R, Postma PW. Autoregulation of lactose uptake through the LacY permease by enzyme IIA(Glc) of the PTS in *Escherichia coli* K-12. *Mol Microbiol*. 1999;31:1825–1833.
23. Wong P, Gladney S, Keasling JD. Mathematical model of the lac operon: inducer exclusion, catabolite repression, and diauxic growth on glucose and lactose. *Biotechnol Prog*. 1997;13:132–143.
24. Mrotzek C, Anderlei T, Henzler HJ, Büchs J. Mass transfer resistance of sterile plugs in shaking bioreactors. *Biochem Eng J*. 2001;7:107–112.
25. Losen M, Frolich B, Pohl M, Büchs J. Effect of oxygen limitation and medium composition on *Escherichia coli* fermentation in shake-flask cultures. *Biotechnol Prog*. 2004;20:1062–1068.
26. Palmen TG, Nieveler J, Frolich B, Treffenfeldt W, Pohl M, Büchs J. Physiological relation between respiration activity and heterologous expression of selected benzoylformate decarboxylase variants in *Escherichia coli*. *Microb Cell Fact*. 2010;9:76.
27. Scheidle M, Dittrich B, Klinger J, Ikeda H, Klee D, Büchs J. Controlling pH in shake flasks using polymer-based controlled-release discs with pre-determined release kinetics. *BMC Biotechnol*. 2011;11:25.
28. Grossman TH, Kawasaki ES, Punreddy SR, Osburne MS. Spontaneous cAMP-dependent derepression of gene expression in stationary phase plays a role in recombinant expression instability. *Gene*. 1998;209:95–103.
29. Mason CA, Bailey JE. Effects of plasmid presence on growth and enzyme-activity of *Escherichia coli* Dh5-Alpha. *Appl Microbiol Biotechnol*. 1989;32:54–60.
30. Valenzuela MS, Ikpeazu EV, Siddiqui KAI. *E. coli* growth inhibition by a high copy number derivative of plasmid pBR322. *Biochem Biophys Res Commun*. 1996;219:876–883.
31. Blommel PG, Becker KJ, Duvnjak P, Fox BG. Enhanced bacterial protein expression during auto-induction obtained by alteration of lac repressor dosage and medium composition. *Biotechnol Prog*. 2007;23:585–598.
32. Jacoby GA. AmpC beta-lactamases. *Clin Microbiol Rev*. 2009;22:161–182.
33. Büssow K, Scheich C, Sievert V, Harttig U, Schultz J, Simon B, Bork P, Lehrach H, Heinemann U. Structural genomics of human proteins—target selection and generation of a public catalogue of expression clones. *Microb Cell Fact*. 2005;4:21.
34. Terpe K. Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Appl Microbiol Biotechnol*. 2006;72:211–222.
35. Donovan RS, Robinson CW, Glick BR. Review: optimizing inducer and culture conditions for expression of foreign proteins under the control of the lac promoter. *J Ind Microbiol*. 1996;16:145–154.
36. Hoffman BJ, Broadwater JA, Johnson P, Harper J, Fox BG, Kenealy WR. Lactose fed-batch overexpression of recombinant metalloproteins in *Escherichia coli* Bl21(De3): process-control yielding high-levels of metal-incorporated, soluble-protein. *Protein Expr Purif*. 1995;6:646–654.
37. Berrow NS, Büssow K, Coutard B, Diprose J, Ekberg M, Folkers GE, Levy N, Lieu V, Owens RJ, Peleg Y, Pinaglia C, Quevillon-Cheruel S, Salim L, Scheich C, Vincentelli R, Busso D. Recombinant protein expression and solubility screening in *Escherichia coli*: a comparative study. *Acta Crystallogr D Biol Crystallogr*. 2006;62:1218–1226.

Manuscript received May 12, 2011, and revision received Aug. 18, 2011.