Scale-up of a Type I secretion system in E. coli using a defined mineral medium

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Abstract
Secretion of heterologous proteins into the culture supernatant in laboratory strains of Escherichia coli is possible by utilizing a Type I secretion system (T1SS). One prominent example for a T1SS is based on the hemolysin A toxin. With this system, heterologous protein secretion has already been achieved. However, no cultivations in a defined mineral medium and in stirred tank bioreactors have been described in literature up to now, hampering the broad applicability of the system. In this study, a mineral medium was developed for cultivation under defined conditions. With this medium, the full potential and advantage of a secretion system in E. coli (low secretion of host proteins, no contamination with proteins from complex media compounds) can now be exploited. Additionally, quantification of the protein amount in the supernatant was demonstrated by application of the Bradford assay. In this work, host cell behavior was described in small scale by online monitoring of the oxygen transfer rate. Scalability was demonstrated by stirred tank fermentation yielding 540 mg/L HlyA1 in the supernatant. This work enhances the applicability of a protein secretion system in E. coli and paves the way for an industrial application.

KEYWORDS
E. coli, metabolic burden, mineral medium, online monitoring, protein secretion, scale-up

1 | INTRODUCTION

The secretion of proteins into the culture medium or the periplasmic space offers several advantages compared to a classic intracellular production. The protein can be obtained in correctly folded form and the purification is simplified compared to purification from lysed cells or inclusion bodies.1 Different strategies can be distinguished to achieve the extracellular localization of a protein using gram-negative bacteria: Active transport into the periplasm, followed by passive transport across a leaky or permeabilized outer membrane is one approach.2 The increasing permeability of the outer membrane can be realized by different strategies, for example, the use of so-called “leaky mutants,” which have mutations in the membrane or cell wall structure.2 It might also be obtained by co-expression of the kil gene that causes the release of periplasmic proteins.3 Besides passive transport across the outer membrane, the use of a secretion system can directly target the protein of interest into the culture supernatant, thereby avoiding the necessity for outer membrane disruption, which might result in a decreased cell viability.2

Among the different secretion systems present in bacteria2 the Type I secretion system (T1SS) is common in a large number of gram-negative bacteria (for reviews covering different aspects of the system see Delepelaire5 and Holland et al.6). The secretion system of the hemolysin A (HlyA) toxin in uropathogenic Escherichia coli belongs to
this class of secretion systems and is one of the most well studied systems.7,8 Secretion takes place in an unfolded state8,10 in one step across both membranes.11-13 Folding into the biologically active conformation is induced in the extracellular space through the binding of calcium ions.14 The transport complex itself consists of three membrane proteins15,16: A membrane fusion protein, HlyD, and an ABC-transporter, HlyB, are both located in the inner membrane while an outer membrane protein, TolC, is located in the outer membrane.17 A so-called secretion signal HlyA1, consisting of the C-terminal 218 amino acids of the full-length toxin, contains all information necessary for secretion and is secreted as efficient as the full-length toxin.18 Consequently, HlyA1 has to be fused to a target protein to achieve its secretion.9

Most of the studies addressing the HlyA secretion system focus on examining the functionality of the transport complex and a proof-of-concept for heterologous protein secretion.19-21 Consequently, most of the experiments in this context have been carried out in complex medium9,20-25 using shake flasks and verification of the product and its concentration are usually determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). However, utilization of a mineral medium is crucial to facilitate process development. First, the valuable advantage of rather “clean” supernatants of E. coli with low impurity and foreign protein content would be exploited, because no foreign proteins and amino acids (originating, e.g., from yeast extract) are present in a mineral medium. Second, product purification and, thus, downstream processing, which is a major cost determinant,26 would be simplified. In addition, foam formation during fermentation in a bubble-aerated system (stirred tank reactor) is reduced, if less foreign proteins and peptides are present in the medium.27 Utilization of a mineral medium enables the cultivation under defined conditions, thereby avoiding lot-to-lot deviations of complex medium components, which might influence the heterologous protein yield.28 Furthermore, quantification of the product (e.g., Bradford measurement) would be possible, which is important for bioprocess development and optimization.

One important aspect associated with recombinant protein production is the so-called metabolic burden (also referred to as “metabolic load” or “metabolic drain”) that arises from introducing foreign DNA into a host cell.29 It is defined as the draining of metabolic resources (building blocks such as amino acids and ATP) from the host cell for the production of a recombinant protein.29,30 By applying the respiration activity monitoring system (RAMOS), the metabolic state of a culture can be assessed.31,32 The system measures the respiration activity in shake flasks using oxygen partial pressure sensors in the gas headspace to calculate the oxygen transfer rate (OTR). Differential pressure sensors are used to calculate the respiratory quotient and the carbon dioxide transfer rate.31,32 RAMOS can be used to evaluate the metabolic burden of a host cell.33

Previous successes in biochemical characterization, improvement of titers, and engineering of the HlyA secretion system have laid the foundation for an industrial application of the system. This study focuses on the scale-up of a fermentation process for the HlyA1 secretion system in E. coli utilizing a defined mineral medium. First, cultivation in a suitable mineral medium is established. Afterward, a first improvement of HlyA1 titers is carried out in shake flasks by online monitoring of the OTR. This way, the metabolic burden of the host cell during secretion is evaluated. Next, scale-up from shake flask to stirred tank is performed. Bradford assay is established for product quantification.

2 | MATERIALS AND METHODS

2.1 | Microorganisms, expression vectors, and target protein

E. coli Tuner strains were purchased from Novagen and transformed with the plasmids depicted in Figure 1. The secretion signal HlyA1 (~23 kDa) is used as a model product in this study and coded on an arabinose-inducible plasmid (“pSOI”) as described by Bakkes et al.9 (“product”) (Figure 1, left). The transport complex proteins HlyB and HlyD are coded on a second plasmid with pK184 backbone as described by Bakkes et al.9 (“transporter”) (Figure 1, right). TolC is endogenously expressed in E. coli24 with an estimated copy number of 1,500 per cell25 and thus, not additionally expressed on a plasmid.

2.2 | Cultivation media and cultivation conditions

All cultivation media were supplemented with the appropriate antibiotics to ensure plasmid stability. The antibiotics were added from sterile filtered stock solutions. 100 µg/ml of ampicillin and 30 µg/ml of kanamycin were used as selection markers for the product plasmid and the transporter plasmid, respectively. All cultivations in shake flasks were performed in 250 ml shake flasks with a filling volume of 10 ml. An incubator shaker (model ISFX-1), purchased from Kuhner AG, Switzerland, with a shaking frequency of 350 rpm, and a shaking diameter of 50 mm was used for all experiments.

2.3 | Complex cultivation media

The cultivation in complex medium was carried out in 2xYT-medium containing 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, and 10 mM calcium (supplemented as CaCl2 ⋅ 2 H2O). The pH was adjusted to 7.0 ± 0.2 using 5 M sodium hydroxide solution. Afterward, the medium was sterilized at 121°C for 20 min.

2.4 | Mineral cultivation media

The cultivation in mineral medium was carried out in modified Wilms-MOPS mineral medium according to Wilms et al.26 The medium consisted of 7.5 g/L glycerol, 6.98 g/L (NH4)2SO4, 3 g/L K2HPO4, 2 g/L Na2SO4, 41.85 g/L (=0.2 M) (N-Morpholino)-propanesulfonic acid (MOPS), 0.5 g/L MgSO4 ⋅ 7 H2O, 0.01 g/L thiamine hydrochloride, 0.1 g/L ampicillin (product plasmid), 0.03 g/L kanamycin (transporter plasmid), 1 ml/L trace element solution [0.54 g/L ZnSO4 ⋅ 7 H2O, 0.48 g/L CuSO4 ⋅ 5 H2O, 0.3 g/L MnSO4 ⋅ H2O, 0.54 g/L CoCl2 ⋅ 6 H2O, 41.76 g/L FeCl3 ⋅ 6 H2O, 1.98 g/L CaCl2 ⋅ 2 H2O, 33.4 g/L Na2EDTA (Titriplex III)]. The pH-value was adjusted to 7.3 using 5 M NaOH. All components were mixed from sterile stock solutions that were...
separately sterilized by autoclaving or sterile filtration. The medium was prepared directly before use. The concentration of calcium (supplemented as CaCl₂ ∙ 2H₂O) and citrate (supplemented as sodium citrate) was 10 mM each. Both were added as the last components from sterile filtered stock solutions. Induction was performed by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG; 1 M stock solution) and/or 10 mM L-(+)-arabinose (2.5 M stock solution) during the cultivation. For the fermentation in stirred tank reactor, MOPS-buffer was omitted from the medium.

2.5 | Cultivation with online monitoring and offline sampling

The first pre-culture was prepared in 2xYT-medium (see above). Ten milliliters of medium were inoculated with 100 μl from a cryo culture (OD₆₀₀ nm, Start = 0.035) that had been stored at −80°C. The pre-culture was incubated for 16 hr (for inoculation of the main culture in complex medium) or for 3–4 hr (for inoculation of the second pre-culture in mineral medium) at 37°C.

For experiments in complex medium, the main culture in 2xYT-medium was inoculated from the first pre-culture with an initial OD₆₀₀ nm of 0.1. For experiments in mineral medium, the second pre-culture in Wilms-MOPS-medium was inoculated from the first pre-culture with an initial OD₆₀₀ nm of 0.01 and cultivated at 30°C for 16 hr. The main culture in Wilms-MOPS-medium was inoculated from the second pre-culture with an initial OD₆₀₀ nm of 0.1. For the inoculation of the main culture in stirred tank fermentation, two flasks of the second pre-culture were run in parallel and pooled prior to measurement of the optical density.

The OTR of the main culture (complex or mineral) was monitored by applying the RAMOS built in-house. For monitoring of the OTR, modified 250 ml shake flasks with a filling volume of 10 ml were used. Measurement and calculation of the OTR was carried out as described by Anderlei et al.31,32 In brief, the partial pressure of oxygen in the headspace is monitored by an oxygen sensor. The oxygen partial pressure in the headspace decreases due to respiration of the microorganisms, because the flask is closed by valves during the measurement phase. Afterward, the flask is flushed with air until the next measurement phase begins. From the decrease of the oxygen partial pressure, the OTR is calculated. Due to the very low changes in dissolved oxygen during batch cultivation, the OTR can be assumed to be equal to the oxygen uptake rate.37 For parallel offline sampling, conventional Erlenmeyer flasks, equipped with a cotton plug, were cultivated at the same conditions as the RAMOS-flasks. At each sampling point, the whole volume of one flask was used for offline determination of the parameters described under "Offline analysis."

2.6 | Fermentation in stirred tank reactor

For the fermentation in a stirred tank reactor, the pre-culture and media preparation were carried out as described in Sections 2.4–2.6. A 2-L Sartorius BIOSTAT® Bplus fermenter (Sartorius, Goettingen, Germany) with a reactor diameter of 13 cm and a filling volume of 1 L was used. The fermenter was equipped with one 6-blade Rushton turbine (stirrer diameter 5 cm) positioned at a height of 0.375 Dₚ. At the beginning of the fermentation, the stirrer speed was set to 500 rpm, the gas flow rate to 60 L/hr (1 vvm), and the pH-value to 6.8. The temperature was set to 37°C for the whole fermentation. During the process, the pH-value was measured with an EasyFerm Plus K8 200 pH sensor (Hamilton, Hoechst, Germany) and controlled at 7.0. It was adjusted by addition of 2 M hydrochloric acid and 25% (wt/vol) ammonia, respectively. The dissolved oxygen tension was measured with an OxyFerm™ FDA 225 pO₂ electrode (Hamilton, Hoechst, Germany). The DOT was maintained at a minimum of 30% by a cascade controller. First, the gas flow rate was increased to 99 L/hr (1.65 vvm), then the stirrer speed was adjusted automatically as needed. After 10 hr, the gas flow rate was increased to 108 L/hr (1.8 vvm). The exhaust gas composition was measured by a DASGIP GA4 off gas analyzer (DASGIP, Juelich, Germany). Antifoam agent Plurafac LF 1300 (BASF SE, Ludwigshafen, Germany) was added when necessary. All measurement values were recorded at an interval of 30 s. To simplify reading of the data, the average of 10 measurement values was calculated and plotted.
2.7 | Offline analysis

2.7.1 | Optical density

The optical density was determined with a spectrophotometer (Genesys 20, Thermo Fisher Scientific, Waltham, MA) at a wavelength of 600 nm in 1 cm cuvettes. The sample was appropriately diluted using 0.9% NaCl (mineral medium) or 2xYT-medium (complex medium). NaCl and 2xYT-medium in the respective dilution were used as blanks. The measurement was carried out in duplicates.

2.7.2 | pH-value

The pH-value was determined with an InLab Easy pH electrode (Mettler Toledo, Germany) that was calibrated using the appropriate calibration buffers and a CyberScan pH 510 meter (Eutech Instruments, Thermo Scientific, Germany).

2.7.3 | Sodium dodecylsulfate polyacrylamide gel electrophoresis

SDS-PAGE was carried out using 12-well NuPAGE® 4–12% Bis-Tris gradient gels (Thermo Fisher Scientific). The sample volume was 20 μl for the supernatant and 10 μl for the cell extract, respectively. For Figure 4, 10 μl of supernatant and cell extract was loaded on a 17-well NuPAGE® 4–12% Bis-Tris gradient gel. Two milliliters of culture broth was centrifuged at 14,000 rpm for 5 min. Afterward, 45 μl of the supernatant was mixed with 15 μl of fourfold concentrated NuPAGE® LDS Sample Buffer (Thermo Fisher Scientific). The cell pellet was washed once with 50 mM phosphate buffer (pH 7.0) and resuspended to a final OD of 5. Ten microliters of PageRule™ unstained protein ladder (Thermo Fisher Scientific) was used as a marker. Sample preparation, loading, running, staining, and destaining were carried out according to the manufacturer’s guidelines.

The gels were scanned using a scanner (Epson Perfection V700 Photo) equipped with the appropriate software (Epson, Japan) and standard settings for highlight (246), shadow (18), and gamma (1.00) levels. Whenever possible and as stated above, the same sample amount and the same scanning options were chosen for all gels to enable a qualitative comparison of the gels and to avoid distortion of the results.

2.7.4 | Bradford assay

The protein concentration in the supernatant was determined in triplicates by Bradford assay using bovine serum albumin (Lot-No. BAH65-882, VWR) as a standard for calibration. The assay was carried out as suggested by the manufacturer (Thermo Fisher Scientific) in 96-well micro titer plates using Coomassie protein assay reagent (Merck, Germany). The values were corrected for evaporation (in shake flasks) and the value for pure medium was subtracted.

2.7.5 | Carbon sources

The concentration of the different carbon sources and media ingredients (glycerol, arabinose, and citrate) was determined via HPLC analysis. An organic acid resin column (250 x 8 mm, CS Chromatographic Services, Germany) was used for separation at a temperature of 80°C and a flow rate of 0.8 ml/min. As a mobile phase, 5 mM H₂SO₄ was used.

3 | RESULTS AND DISCUSSION

3.1 | Protein secretion in complex medium

Since for many applications a tightly controlled and well inducible protein expression is desired, an arabinose-inducible product plasmid (pBAD-promotor) was investigated. The transporter (HyB and HyD) remained on a different plasmid under control of a lac promoter (see Figure 1). Both promoters are subject to “all-or-none behavior.”

This means, that a fraction of cells is fully induced, while another fraction might not be induced at all. Therefore, E. coli Tuner was used as host strain. In contrast to the commonly used BL21(DE3) strain, the Tuner strain carries a mutation in the lac-permease, allowing a homogenous induction of the lac promoter with IPTG.

A concentration of 1 mM IPTG was chosen for comparison with previous studies utilizing the same transporter plasmid. For arabinose, a concentration of 10 mM was used to prevent the inhibition of the araBAD-promoter by IPTG. IPTG was added at the beginning of the cultivation to allow the expression and assembly of the transport complex prior to the expression of HlyA1. The OTR over time and the SDS-PAGE analysis are depicted in Figure 2. It should be noted that the OTR values directly after inoculation (first hour of the cultivation) are inaccurate, because the measurement of the OTR is very sensitive toward temperature changes. After inoculation, sometime (about 1 hr) is needed for the liquid inside the flask to reach the same temperature as the incubator.

After induction with arabinose, the OTR of the induced culture (open stars) differs from the culture that has not been induced (closed stars), staying on a lower OTR-level. SDS-PAGE analysis (Figure 2b) shows that HlyA1 (23 kDa) is well secreted, if arabinose was added to the cultivation (open star). In the absence of arabinose, the expression of HlyA1 is not induced and, consequently, no product band is detectable on the SDS-gel. The experiment was replicated (see Figure A1). With the constraint of a deviation in the absolute values of the OTR and differences in the length of the lag-phase, the results are well reproducible, especially with regard to the secreted amount and localization of HlyA1.

3.2 | Mineral medium for protein secretion in E. coli

In a next step, a defined mineral medium was adapted for the cultivation with this secretion system. To the author’s knowledge, no cultivations have been performed in defined mineral medium with this secretion system, yet. A reason for this might be that previous studies...
Cultivation conditions: 250 ml shake flask with 10 ml filling volume, (pSOI with HlyA1) with 10 mM arabinose after 1.5 hr and transporter IPTG at the beginning (open stars). Induction of the product plasmid time. Induction of the transporter (pK184 with HlyBD) with 1 mM medium. (a) Oxygen transfer rate (OTR) as function of the cultivation time. Induction of the transporter (pK184 with HlyBD) with 1 mM IPTG at the beginning (open stars). Cultivation conditions: 250 ml shake flask with 10 ml filling volume, shaking frequency 350 rpm, temperature 37°C, shaking diameter 50 mm, 2xYT-medium with 10 mM CaCl₂. (b) SDS-PAGE after 7 hr of cultivation. M = Protein standard, lanes 1 + 2: Supernatant and cell extract of E. coli Tuner induced with IPTG, lanes 3 + 4: Supernatant and cell extract of E. coli Tuner induced with IPTG and arabinose. HlyA1 has an expected size of ~23 kDa. Dashed line indicates where the gel image was cut and rearranged. Original gel image is shown in Figure A6. IPTG, isopropyl-β-D-thiogalactoside; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis focused on the biochemical characterization of the system (see Section 1) with a minor focus on a future industrial application. Another reason might be that high calcium concentrations are required for product stabilization, which tend to precipitate as calcium phosphate or calcium sulfate for example.

The mineral medium is based on a medium developed by Wilms et al.36 for the cultivation of E. coli and was adapted as mentioned in Section 2.4. Glycerol was chosen as a carbon source that, in contrast to glucose, neither inhibits the transcription from the lac- nor the araBAD-promoter. Since 10 mM calcium were added in complex medium, the same concentration was chosen for the mineral medium.

Figure 3a depicts the course of the OTR for different inducer combinations. There is only a minor difference in the OTR between the cultures that were not induced (closed diamonds) or induced only with IPTG (closed triangles) (Figure 3a, top diagram). This might be caused by slight differences in initial biomass concentration, as the cultivations were performed in subsequent experiments. As can be seen by comparison of the OTR (closed triangles) and the glycerol concentration (closed squares) for the culture induced with 1 mM IPTG, the OTR increases exponentially until all glycerol is depleted. Consequently, the optical density (open stars) also increases exponentially. In contrast to that, induction with 10 mM (=1.5 g/L) arabinose leads to a phase of constant OTR after induction (Figure 3a, middle diagram, open circles and closed squares), which is followed by a slight kink after around 9 hr. Afterward, the OTR increases exponentially until glycerol is depleted. This behavior is independent of the presence of IPTG. The offline data for the culture induced with 10 mM (=1.5 g/L) arabinose and IPTG (Figure 3a, bottom diagram) prove that the cultivation can be divided into four different phases that are already visible from the OTR signal itself. In the first 3 hr (Phase I) the cells grow non-induced on glycerol (Figure 3a, bottom, open squares). In this phase no expression and secretion of HlyA1 occurs (Figure 3b,c). During Phase II, the optical density (Figure 3a, bottom diagram, closed stars) increases only slightly and arabinose (Figure 3a, bottom diagram, open triangles) is consumed. The amount of HlyA1 in the supernatant increases over time (Figure 3b). This “induction phase” is reflected by a constant level of the OTR. The phenomenon of a more or less constant OTR after induction is well known from auto-induction media containing glucose, lactose, and glycerol. Several studies identified a phase of rather constant OTR after induction with IPTG or its analogue lactose as production phase and indication of the strength of metabolic burden.33,44,45 An equivalent behavior is shown here for the first time in a mineral medium using arabinose as inducer. The reason for this observation (more or less constant OTR after induction) remains unclear and was observed for a single amino-acid exchange,33 different host strains expressing the same product45 as well as a single nucleotide exchange.46 In all cases, biomass formation declined upon induction, which is in agreement with data from literature and a result of a higher ATP demand.46 The increased ATP demand is, however, not fulfilled by means of oxidative phosphorylation as the oxygen demand is constant after induction. Thus, another explanation might be that the cells use substrate level phosphorylation to supply ATP instead of oxidative phosphorylation. As the metabolic burden response affects a broad range of cellular functions, a combination of different regulatory mechanisms might also be responsible for this observation. Overall, it is rather difficult to give one overall explanation for the observed phenomenon without a thorough biochemical analysis. This was, however, not part of this study, but should be investigated in more detail in the future.

As soon as arabinose is depleted, a distinct kink can be identified in the OTR (Figure 3a, Phase III). Most likely it appears due to changes in the cell metabolism (diauxic rearrangement of metabolism).32 Afterward, cell growth continues, as indicated by an exponential increase in optical density and OTR. The remaining glycerol is consumed in a second non-induced growth phase (Phase IV). During this phase, the amount of HlyA1 in the supernatant slightly decreases, which was verified by Bradford measurement of the protein concentration in the supernatant (Figure 3a, bottom diagram, closed circles). In parallel, the HlyA1 concentration simultaneously increases in the cell extract fraction (Figure 3c).

According to literature, intracellularly produced HlyA1 is rapidly degraded.17 Thus, the model product HlyA1 that is found in the cell extract fraction has probably been secreted and subsequently precipitated from the supernatant. Protein stability in the supernatant might...
be affected by many parameters like ammonium concentration in the medium, pH-value (see Kramer et al.47 for a comprehensive review) and exposure to oxygen radicals.48 The process of protein precipitation is still poorly understood. Since the reason for the instability of the product in shake flasks remains unclear, it is subject of further investigations. Furthermore, a degradation band appears after around 11 hr with a molecular weight of ~20 kDa (Figure 3b). Since the induction phase ends after about 9.5 hr at the end of Phase III (Figure 3a, bottom diagram), the cultivation should be terminated at this point. As soon as glycerol is depleted, the OTR drops to very low levels, indicating the end of active respiration.

Figure 3d depicts the corresponding SDS-gels for the graphs shown in the upper diagram of Figure 3a. As expected, no product is present in the supernatant, if no inducer ("w/o inducer") or only IPTG ("IPTG") has been added to the culture. In contrast to this, HlyA1 is secreted into the supernatant in equal amounts for cultures induced with IPTG and arabinose ("IPTG, arabinose") or only with arabinose ("arabinose"). The results were reproduced in a consecutive experiment (Figure A2).

Full secretion in presence and absence of IPTG indicates that the transporter is sufficiently expressed in the absence of IPTG. Cultivation without the transporter present proves that the transporter plasmid (HlyB and HlyD on pK184-plasmid with lac promotor) is needed for secretion (Figure A3b). This is also reflected in the respiration profile (no constant OTR after addition of arabinose) (Figure A3a). In contrast to arabinose, the addition of IPTG itself does not place a metabolic load
on the cells (Figure 3a, closed triangles and closed diamonds). From an industrial point of view, omitting IPTG for induction is an advantage, which reduces costs. However, to enable comparison with previous studies and data presented within this work, all other experiments were performed with addition of IPTG. In future work, experiments will be performed without the initial addition of IPTG for a comparison with the ones with IPTG.

3.3 Variation of the time of induction

Time of induction and inducer concentration is known to have a high impact on recombinant protein expression. Since secretion of HlyA1 is well controlled by addition of arabinose, time of induction and inducer concentration were varied to study the effect on the respiration behavior of E. coli and the HlyA1 concentration in the supernatant. Variation of the inducer concentration (10–50 mM arabinose) did not result in increased amounts of HlyA1 in the supernatant (data not shown). The variation of time of induction was investigated. In addition to an induction at an OTR of 5 mmol/L/hr after 3 hr (Figure 3), an OTR of 17 mmol/L/hr, representing the mid-exponential growth phase, was chosen as an induction point. To enable comparison with subsequent experiments, the induction was performed at a defined value of the OTR instead of a specific cultivation time. This way, slight differences in the cultivation (e.g., different initial biomass concentration) are compensated. The OTR of reproducible duplicates is depicted in Figure 4.

With the information from Figure 3 (phases), it becomes obvious, that arabinose is depleted after around 9 hr of cultivation, independently of the time of induction. This can again be interpreted from the short drop in the OTR (diauxic rearrangement of metabolism) followed by an increase in the OTR beyond the level during the induction phase in both cases. Afterward, an exponential increase in the OTR is caused by the consumption of the remaining glycerol. As expected, the later time of induction (Figure 4, closed triangles) results in a higher amount of biomass at the time of induction (data not shown). This is also represented by a higher OTR. Consequently, the induction phase is shorter (more cells are consuming the arabinose). Thus, the non-induced second growth phase is less distinct, because more glycerol had been consumed before the induction was performed and less glycerol remained for the second non-induced growth phase. Therefore, the overall cultivation time is shorter for the later induced culture. Nevertheless, the same phases that have already been identified and described in Figure 3 can be assigned to this culture. The course of the OTR is well reproducible (Figure A4). Figure 4b shows the SDS-gel at the point where arabinose was depleted (9 hr). While there is again some protein present in the cell extract (closed square and triangle on the right), there clearly is a higher amount of product secreted when the induction was performed after 5 hr (closed square and triangle on the left). Measurement by Bradford assay shows that the protein concentration was increased from 190 (±11) mg/L to 621 (±67) mg/L. This corresponds to a more than threefold increase, which is also reflected (qualitatively) in the SDS-PAGE (Figure 4b). In addition, a sample was also taken from a RAMOS flask for comparison with subsequent experiments. The OTR of reproducible duplicates is shown in Figure 4.

For both cultures presented in Figure 4, a slight degradation band is visible below the target protein band. Depending on the target application of the product, this might necessitate further purification (if the product is, e.g., intended for medical applications) or is tolerable (if the product is, e.g., a technical enzyme). However, compared to an intracellularly produced protein, which necessitates opening of the cell envelope and releasing all host proteins, the purification process is much simplified.

3.4 Scale-up into stirred tank bioreactor

A protein that is secreted into the supernatant might experience a different environment in a stirred tank reactor, in comparison to a shake flask. Due to different aeration (surface aeration vs. bubble aeration) and agitation (shaking vs. stirring), scale-up from shake flask to stirred tank reactor is a crucial step for process development. It is also a prerequisite for an industrial application. To prove that the production of the secretion signal and model product HlyA1 is also possible in a stirred tank bioreactor, a scale-up was performed using the adapted mineral medium. The pH and temperature were kept constant and the DOT was kept above...
30% during cultivation (Figure A5). The results of the OTR and offline samples are shown in Figure 5.

The course of the OTR (Figure 5a, upper diagram) is similar to the course of the OTR in shake flasks (see, e.g., Figure 4). The different cultivation phases already identified by monitoring of the OTR in shake flasks (Section 3.2) can be recognized in stirred tank as well. Arabinose was added after 6.75 hr in stirred tank to induce the culture at a similar OTR as in shake flasks (Figure 4). The product concentration in the supernatant increases as soon as arabinose has been added (Figure 5a, lower diagram and Figure 5b). A final HlyA1 product concentration of 540 mg/L is reached after 11 hr of cultivation. This is about 13% lower compared to an induction at an OTR of 17 mmol/L/hr in shake flasks. A reason for this might be attributed to slight precipitation of HlyA1 from the supernatant. However, it should be noted that the fermentation should be terminated after depletion of arabinose. This way, the space–time-yield is maximized and the down time of the reactor is kept as short as possible. Both aspects are especially important in an industrial production process.

In contrast to the cultivation in shake flasks (Figure 3), no protein accumulates in the cell extract during cultivation in stirred tank. Only after arabinose is already depleted, a slight decrease of HlyA1 is observed in the supernatant (Figure 5a, open circles). Since the course of the OTR is similar in shake flasks and stirred tank, the respiration activity is not affected by the different localization of the product. This supports the hypothesis that HlyA1 was secreted, but subsequently precipitated from the supernatant (see Section 3.2). The reason for the partly different localization in shake flask and stirred tank is unclear and subject to further investigations. Besides differences in aeration and agitation (see above), it might be caused by differences in hydromechnical stress.51,52

FIGURE 5  Cultivation of E. coli Tuner secreting HlyA1 in stirred tank. (a) Upper diagram: Oxygen transfer rate (OTR, closed squares) and model product (HlyA1) concentration (open circles) in the supernatant over time for E. coli Tuner model product (HlyA1) on arabinose-inducible pSOI-plasmid and HlyBD on pK184-plasmid. Induction with 1 mM IPTG at the beginning and with 10 mM arabinose after 6.75 hr. Lower diagram: Offline data (glycerol [open squares], optical density [closed stars], and arabinose [open triangles] over time). Cultivation conditions: 2 L stirred tank reactor with 1 L filling volume, Stirrer speed: 500–950 rpm, gas flow rate: 60–108 L/min (1–1.8 vvm), temperature: 37°C, pH: 6.8–7.0, minimum dissolved oxygen tension: 30% (adjusted automatically by stirrer speed and gas flow rate), 300 μl antifoam agent were added after 6.8 hr, Mineral medium (7.5 g/L glycerol, 10 mM CaCl2). Determination of protein concentration in triplicate by Bradford assay with BSA as calibration standard. (b) and (c) SDS-PAGE of supernatants (b) and cell extracts (c) over time. M = Protein standard.
CONCLUSION

This study for the first time demonstrates the scalability of a system based on the HlyA secretion system in E. coli. A mineral medium was successfully applied for the cultivation under defined conditions. Full exploitation of the system’s advantages compared to other bacterial secretion systems and previous studies (“clean” supernatants) was achieved this way. Measurement of the OTR in shake flasks was applied to investigate the host cell behavior and increase the HlyA1 (model product) concentration prior to scale-up. Scale-up from shake flask to stirred tank was successfully demonstrated. In addition, quantification of the protein concentration in the supernatant by Bradford assay was successfully applied.

Tight controlled secretion of the model product HlyA1 was achieved in complex and mineral medium by using the arabinose-inducible product plasmid pSOI. In mineral medium, the addition of arabinose led to a phase of more or less constant OTR after induction. Induction with 10 mM arabinose at an OTR of 17 mmol/L/hr resulted in a threefold increase in the product concentration in the supernatant compared to an induction at an OTR of 5 mmol/L/hr. Product concentrations after depletion of arabinose were 621 and 190 mg/L, respectively. Scale-up from shake flask to stirred tank was carried out in batch cultivation. A maximum of 540 mg/L HlyA1 was produced in stirred tank and the same respiration profile as in shake flasks was observed.

The full potential of the system will now be investigated by secretion of different products. Furthermore, additional plasmid systems (e.g., T7-system) will be tested. A mathematical method was recently developed to predict the expression performance of different E. coli strains cultured under different conditions, based on the course of the scattered light signal over time. This approach might in the future be used to evaluate the secretion performance of different products (e.g., enzymes) in the presented secretion system.

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CONFLICT OF INTEREST

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