A novel E. coli strain shows controllable leakiness for extracellular production of recombinant proteins

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Abstract

Recombinant proteins in Escherichia coli are usually expressed inside the cell. With the growing interest in continuous cultivation, secretion of product to the medium is not only a benefit, but a necessity in future bioprocessing. In this study, we present the X-press strain, a novel E. coli production host for growth decoupled, extracellular recombinant protein production. We investigated the effect of the process parameters temperature and specific glucose uptake rate (qS) on the strain's growth, productivity, lysis and leakiness, to find the parameter space allowing extracellular protein production. Two model proteins were used, Protein A and a VHH single-domain antibody, and performance was compared to the industrial standard strain BL21(DE3). We show that inducible growth repression in the X-press strain greatly mitigates the effect of metabolic burden under different process conditions. Furthermore, temperature and qS were used to control productivity and leakiness. In the X-press strain, extracellular Protein A and VHH titer reached up to 349 mg/g and 19.6 mg/g, respectively, comprising up to 90% of total soluble product, while keeping cell lysis at a minimum. Our findings demonstrate that the X-press strain constitutes a valuable host for extracellular production of recombinant protein with E. coli.

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Recombinant proteins in *Escherichia coli* are usually expressed inside the cell. With the growing interest in continuous cultivation, secretion of product to the medium is not only a benefit, but a necessity in future bioprocessing. In this study, we present the X-press strain, a novel *E. coli* production host for growth decoupled, extracellular recombinant protein production. We investigated the effect of the process parameters temperature and specific glucose uptake rate (q_S) on the strain's growth, productivity, lysis and leakiness, to find the parameter space allowing extracellular protein production. Two model proteins were used, Protein A and a VHH single-domain antibody, and performance was compared to the industrial standard strain BL21(DE3). We show that inducible growth repression in the X-press strain greatly mitigates the effect of metabolic burden under different process conditions. Furthermore, temperature and q_S were used to control productivity and leakiness. In the X-press strain, extracellular Protein A and VHH titer reached up to 349 mg/g and 19.6 mg/g, respectively, comprising up to 90% of total soluble product, while keeping cell lysis at a minimum. Our findings demonstrate that the X-press strain constitutes a valuable host for extracellular production of recombinant protein with *E. coli* .

Keywords

continuous manufacturing; leakiness; outer membrane integrity; periplasmic protein release; secretion

Introduction

Escherichia coli is a widely used expression host for recombinant protein production. Its advantages lie in short doubling times, growth on cheap media to high cell densities and straightforward cloning procedures (Kleiner-Grote, Risse, & Friehs, 2018; Rosano & Ceccarelli, 2014; Yoon, Kim, & Kim, 2010). However, the product is usually expressed inside the cell, which requires cell disruption in downstream processing, leading to release of unwanted host cell proteins and other contaminants, like lipids and DNA (Balasundaram, Harrison, & Bracewell, 2009). If the target protein is produced as insoluble inclusion bodies (IBs), additional IB processing is needed.

Especially with the growing interest in continuous manufacturing (C. Chen, Wong, & Goudar, 2018; Kateja, Agarwal, Hebbi, & Rathore, 2017), extracellular production is an important enabler for future bioprocessing with *E. coli* . Secretion of recombinant protein to the medium furthermore enhances solubility, stability and biological activity of the product (Mergulhão & Monteiro, 2007). This can be achieved either by one-step-secretion (directly from the cytoplasm to the extracellular space) via the T1SS or T3SS system, or by two-step-secretion: In the first step, the protein is directed through the inner membrane via the Sec- or Tat-pathway. In the second step, the outer membrane (OM) is made permeable, or "leaky", to release the product to the medium (Kleiner-Grote et al., 2018). Numerous studies on how to increase leakiness during cultivation exist and several reviews cover this research in detail (Kleiner-Grote et al., 2018; Mergulhão, Summers, & Monteiro, 2005; Yoon et al., 2010).

One approach to increase leakiness is chemical permeabilization by addition of media supplements, like Triton-X, glycine or EDTA. However, those additives usually have detrimental effects on the viability of the cells and might harm the product (Kleiner-Grote et al., 2018). Another approach is the generation of leaky *E. coli* mutants. Many expression systems that show permanently high leakiness have been engineered to date. Their outer membrane structure is usually altered by mutations in cell envelope genes and signal peptides are optimized for higher translocation efficiency (Kleiner-Grote et al., 2018; Zhou, Lu, Wang, Selvaraj, & Zhang, 2018). However, detailed process information at bioreactor scale is often missing for these strains.

Another reported strategy is the enhancement of OM permeability via temperature or specific growth rate (μ). Shokri, Sanden, and Larsson (2002) showed that growth rate dependent changes in the membrane composition have an effect on protein leakage. In their study, in continuous cultivation of *E. coli* W3110, leakiness had an optimum at a dilution rate of 0.3 h^{-1} and declined upon lowering or increasing μ . Similar results were obtained in fed-batch studies of W3110 (Voulgaris, Finka, Uden, & Hoare, 2015) and a K12 derivate (Bäcklund et al., 2008), in which an increase in μled to enhanced leakiness. Contrarily, Rinas and Hoffmann (2004) stated that μ had no significant effect on periplasmic protein release during heat induction of *E. coli* TG1 strains. In another fed-batch study using a C41(DE3) strain, it has even been stated thatμ and leakiness are inversely correlated (Wurm, Marschall, Sagmeister, Herwig, & Spadiut, 2017). Adverse reports can also be found about the influence of temperature on OM leakiness for different*E. coli* strains. While Rodríguez-Carmona et al. (2012) found that leakage of a Fab was enhanced at lower temperatures, several other studies suggest that increased temperature drives periplasmic protein release (Rinas & Hoffmann, 2004; Wurm, Marschall, et al., 2017; Wurm, Slouka, Bosilj, Herwig, & Spadiut, 2017). Controlling leakiness via temperature and μ is an interesting approach, since it does not require alteration of the chemical environment and is easy to implement, however, the contrary results in the aforementioned studies illustrate that the mechanisms that temperature and μ exert on outer membrane leakiness might depend on a variety of factors, like the strain, product or promoter, and are not fully understood yet.

In this study, we investigated the influence of the process parameters cultivation temperature and specific glucose uptake rate (q_S , linked to μ via the biomass yield $Y_{X/S}$) on OM leakiness of a novel *E. coli*expression host. The X-press strain is a proprietary expression technology recently developed by enGenes Biotech GmbH (Mairhofer, Striedner, Grabherr, & Wilde, 2016; Stargardt, Feuchtenhofer, Cserjan-Puschmann, Striedner, & Mairhofer, 2020). It is derived from BL21(DE3) and carries a genomically integrated sequence coding for the bacteriophage-derived RNA polymerase inhibitor Gp2 under control of the*araB* promoter. This protein from the T7 phage inhibits the host RNA polymerase, while the T7 RNA polymerase stays unaffected. Thus, upon

induction with L-arabinose, host mRNA levels and cell proliferation are reduced, while IPTG-induced target protein expression is enhanced. This approach to decouple growth from recombinant protein production has already been shown to increase specific yield and product quality (Lemmerer et al., 2019; Stargardt et al., 2020). In previous experiments, the X-press strain showed high tendency to leak periplasmic protein to the medium (Stargardt et al., 2020). Therefore, in the present research, we further investigated its response to the process parameters temperature and q_S , both known to affect leakiness of other *E. coli*strains, in fed-batch cultivations. We performed a screening Design of Experiments (DoE) to find the adequate parameter space for enhancing leakiness while maintaining high productivity and viability. We compared the X-press strain to the industrial standard strain BL21(DE3), using two industrially relevant model proteins: Protein A (SpA) from*Staphylococcus aureus* and a VHH sdAb (VHH). The processes were analyzed with respect to $Y_{X/S}$, productivity, lysis and leakiness. With this holistic approach, we aimed at 1) characterization of a novel *E. coli* expression host for growth decoupled protein secretion and 2) finding the parameter space that allows tight control of leakiness and productivity for extracellular recombinant protein production.

Materials and Methods

Strains

Two *E. coli* strains were used in this study: the X-press strain, a BL21(DE3) derivate patented by enGenes Biotech GmbH (Mairhofer et al., 2016; Stargardt et al., 2020), and a state-of-the-art BL21(DE3) strain (New England Biolabs, Ipswich, MA). The X-press strain carries a genomically integrated sequence coding for Gp2, a protein repressing cell growth by inhibition of RNA polymerase. Its expression is induced by L-arabinose, which cannot be degraded by X-press due to a knockout of the *araABCD* operon. For determination of cell growth repression solely induced by Gp2 expression, the plasmid free X-press strain was used. For recombinant protein production, both strains were transformed with a pET30a plasmid containing a *cer* sequence for enhanced plasmid stability (Bower & Prather, 2009) and a kanamycin resistance marker. The plasmid carried the gene coding for 1) the IgG-binding domains of Protein A from *Staphylococcus aureus* (SpA) with the pelB signal sequence or 2) the anti-TNFRI VHH single domain antibody DOM101 with the ompA signal sequence (Chatel et al., 2014). Both proteins were His-tagged at the C-terminus. Protein sequences are listed in Supporting Information 1.

Media

The semi-defined medium for the pre-culture contained 9.00 g/L glucose, 3.00 g/L KH_2PO_4, 4.58 g/L K_2HPO_4, 0.30 g/L peptone, 0.15 g/L yeast extract, 0.75 g/L sodium citrate dihydrate, 0.30 g/L $MgSO_4$ [?] $7H_2O$, 0.03 g/L $CaCl_2$ [?] $2H_2O$, 1.35 g/L $(NH_4)_2SO_4$, 1.11 g/L NH_4Cl, 50 mg/L kanamycin and 150 µL/L of a solution containing 40.00 g/L $FeSO_4$ [?]$7H_2O$, 10.00 g/L $MnSO_4$ [?]H_2O, 10.00 g/L $AlCl_3$ [?]$6H_2O$, 7.30 g/L $CoCl_2$ [?]$6H_2O$, 2.00 g/L $ZnSO_4$ [?]$7H_2O$, 2.00 g/L $NaMoO_4$ [?]$2H_2O$, 1.00 g/L $CuCl_2$ [?]$2H_2O$, 0.50 g/L H_3BO_3. For bioreactor cultivations, defined minimal media according to DeLisa, Li, Rao, Weigand, and Bentley (1999) was used, with glucose as carbon source. The initial glucose concentration was 20 g/L and the substrate feed had a glucose concentration of 400 g/L.

Bioreactor cultivations

For the pre-culture, 500 mL of semi-defined medium were inoculated with a frozen stock in a 2500 mL High Yield shake flask and incubated for 16 h at 37°C and 230 rpm in an Infors HR Multitron incubator (Infors, Bottmingen, Switzerland).

The plasmid free X-press strain was cultivated in a stainless steel bioreactor with a working volume of 10 L (Biostat Cplus, Sartorius, Göttingen, Germany). The batch volume was 5 L. The culture broth was supplied with a mixture of air and pure oxygen at 10 L/min and stirred constantly at 1200 rpm. Dissolved oxygen (DO) was monitored using a fluorescence electrode (Visiferm DO120, Hamilton, Reno, NV, USA) and kept above 35% by adjusting the amount of added pure oxygen. pH was monitored with an Easyferm electrode (Hamilton) and kept constant at 7.00 via addition of NH_4OH (12.5%). The temperature was controlled with the built-in heat jacket and kept at 37°C, except during induction (described below).

The recombinant protein production processes were carried out in a DASGIP parallel reactor system (Eppendorf, Hamburg, Germany) with four vessels containing 2 L working volume, aerated at 2 L/min. The batch volume was 1 L. Gas mixing and control of DO, pH and temperature (via heat blanket and cooling finger) were done analogously to the cultivations in the stainless steel bioreactor described above.

The batch was started by inoculating minimal media (90% of the batch volume) with the preculture (10% of the batch volume). Once glucose was depleted (detected by a DO spike), substrate was fed to reach a cell dry weight concentration of 50 g/L and 30 g/L in the growth repression and recombinant protein production processes, respectively. Subsequently, expression of Protein A or VHH was induced by addition of 0.5 mM or 0.25 mM IPTG, respectively. Additionally, Gp2 expression in the X-press strain was induced by adding 100 mM L-arabinose.

Design of Experiments

To study the effect of temperature and q_S on growth, productivity, lysis and leakiness during SpA production, a full-factorial screening DoE was performed. Since growth of the X-press strain is repressed by Gp2 expression during induction, we chose to apply a constant substrate feed rate in our experiments. In the DoE, this is reflected in the first factor as the specific glucose uptake rate with respect to biomass at start of induction ($q_{S,0}$). It was set to 0.13, 0.25 and 0.50 g/g/h, respectively. Temperature during induction, the second factor, was 25, 30 or 35°C, respectively. The different parameter settings are hereafter referred to as "temperature [°C]/glucose uptake rate [g/g/h]", e.g. 30/0.25. Thus, the five factor combinations for the SpA cultivations were: 25/0.13, 25/0.5, 30/0.25/, 35/0.13, 35/0.5. At$q_{S,0}$ = 0.5 g/g/h, the physiological capabilities of the X-press strain were far exceeded (manifested in glucose accumulation and lysis, see Supporting Information 2) and linear regression did not result in significant model coefficients, thus we decided to omit the data of these experiments from further analysis in this study. In addition to the SpA cultivations, the growth arrest experiment with the plasmid free X-press strain was conducted at conditions 30/0.13. Furthermore, the process conditions leading to the highest productivity during SpA cultivations were repeated in both strains containing the plasmid with the VHH sequence.

Analysis of biomass yield

Biomass was quantified gravimetrically in triplicate by centrifuging 2 mL of culture (4000 rcf, 10 min), washing the pellets with 0.9% (w/v) NaCl and drying them at 105°C for 72h. Dry biomass concentration was then determined by weighing the dry pellets. Concentrations of residual glucose and L-arabinose in the cell-free supernatant were analyzed via HPLC (UltiMate 3000; Thermo Fisher, Waltham, MA) with a Supelcogel C-610H column (Supelco, Bellefonte, PA). The eluent was 0.1% H_3PO_4 and the flow rate constant at 0.5 mL/min.

$Y_{X/S}$ was calculated for the time before induction and after induction, respectively (Equations 1 & 2). For this, only the net biomass accumulation, corrected for intracellular product, was taken into account.

$$Y_{X/S,0} = \frac{m_{X,i} - m_{X,0}}{m_{S,i}} \quad (1)$$

$$Y_{X/S,n} = \frac{m_{X,n} - m_{X,i}}{m_{S,n} - m_{S,i}} \quad (2)$$

$Y_{X/S,0}$ is the biomass yield before induction [g/g]; $m_{X,i}$ is the biomass at time of induction [g]; $m_{X,0}$ is the biomass at time of inoculation [g]; $m_{S,i}$ is the consumed sugar at time of induction [g]; $Y_{X/S,n}$ is the biomass yield after induction at time point n [g/g];$m_{X,n}$ is the biomass after induction at time point$n$ [g]; $m_{S,n}$ is the consumed sugar after induction at time point n [g].

Analysis of lysis

Under the assumption that released DNA is proportional to the amount of lysed cells, quantification of lysis was adapted from Klein et al. (2015). For our calculations, we assumed a cellular DNA content of 31 mg/g, which was taken from literature (Neidhardt & Umbarger, 1996). Double strand DNA in the culture supernatant was measured in triplicate with the Quant-iT PicoGreen® dsDNA Assay Kit (Thermo Fisher). The accumulation of DNA was corrected with a degradation rate as described in Supporting Information 3. The amount of lysed cells (x_l) was calculated according to Klein et al. (2015) and expressed as percent of total biomass concentration as shown in Equation 3:

$$lysed\ cells = \frac{x_l}{x_l + x} * 100 \quad (3)$$

x_l , amount of lysed biomass [g/L]; x , cell dry weight [g/L].

Product analysis

For SpA quantification, 10 mL culture were centrifuged for 10 minutes at 15,000 rcf and 4°C. The supernatant was aliquoted and stored at -20°C. The cell pellet was re-suspended in 35 mL of TRIS-buffer (100 mM TRIS, 10 mM EDTA, pH 7.4). This suspension was homogenized in an Emulsiflex C3 homogenizer (Avestin, Ottawa, ON, Canada) (5 passages, 1000 bar) and the sample was then centrifuged for 15 minutes (20,000 rcf, 4°C). The pellet was stored at -20°C. Intracellular soluble SpA content and SpA content in the cell-free culture supernatant were quantified in triplicate by HPLC analysis using a reversed phase column (BioResolve RP mAb Polypheyl; Waters, Milford, MA) and a gradient of acetonitrile and water, both supplemented with 0.1% (v/v) trifluoroacetic acid.

VHH quantification was done analogously, with the exception that the cell pellet was sonicated in MES-Buffer (100 mM MES, 10 mM EDTA, pH 6.0) and HPLC analysis was performed with a cation exchange column (BioResolve SCX mAb; Waters). The loading buffer was 20 mM MES, pH 6.0 and VHH was eluted with a Na^+ gradient.

Inclusion body formation of VHH was analyzed qualitatively by SDS-PAGE. For this, the pellet obtained after homogenization was resuspended in 20 mL of Buffer A (50 mM TRIS, 0.5 M NaCl, 0.02% Tween, pH 8.0) and then centrifuged for 10 minutes (10,000 rcf, 4degC). The resulting pellet was washed in 20 mL Buffer B (50 mM TRIS, 5 mM EDTA, pH 8.0) and 2 mL aliquots were centrifuged for 10 minutes (10,000 rcf, 4degC). Subsequently, the pellet was resuspended in 1 mL ultrapure water, diluted with 1.5 x Laemmli buffer. A VHH standard (5 g/L) was diluted in 2 x Laemmli buffer. The samples and standard were then incubated at 95degC for 15 minutes. 10 µL of sample and 5 µL of standard were loaded onto precast SDS gels (8-16%, Mini-PROTEAN TGX; Bio-Rad, Hercules, CA). Gels were run at 120 V for 30 minutes in a Mini-PROTEAN Tetra-Cell (Bio-Rad) and stained with Coomassie Blue. Images were captured and analyzed using the software Image Lab (Bio-Rad).

Calculation of leakiness

The quotient of soluble extracellular and total intracellular product (leakiness) in percent was calculated using Equation 4:

$$leakiness = \frac{w_{\mathrm{Pin}}}{w_{\mathrm{Pex}} + w_{\mathrm{Pin}}} * 100 \quad (4)$$

w_{Pin} , biomass specific intracellular product concentration [mg/g]; w_{Pex} , biomass specific extracellular product concentration [mg/g].

Results and Discussion

The process parameters temperature and μ are important factors in bioprocess development and are known to have an impact on leakiness of different *E. coli* strains (Table 1). Although conclusive, mechanistic understanding is still missing. In this study, we performed characterization of the novel *E. coli* X-press strain in fed batch cultivations by investigating the influence of temperature and $q_{S,0}$ (q_S at the beginning of induction) on growth, productivity, lysis and leakiness, with the aim of understanding the behavior of the strain and finding a cultivation strategy that allows the successful control of product location. For comparison of the X-press strain to a benchmark strain, we chose *E. coli* BL21(DE3), since it is the most widely used *E. coli* strain for recombinant protein production (Jia & Jeon, 2016; Rosano, Morales, & Ceccarelli, 2019). Three combinations of cultivation temperature and $q_{S,0}$ (30/0.25, 25/0.13, 35/0.13) were first applied using the model protein SpA. The most favorable production conditions were then tested again with the second model protein VHH.

Impact of process parameters on $Y_{X/S}$

It has long been known that heterologous expression in plasmid-based *E. coli* systems has a grave impact on cell physiology, widely known as *metabolic burden* (Mairhofer, Scharl, Marisch, Cserjan-Puschmann, & Striedner, 2013). This burden is often associated with a decrease in growth rate and ultimately cell lysis (Bentley, Mirjalili, Andersen, Davis, & Kompala, 1990; Bienick et al., 2014). We assessed the impact of the selected process parameters on growth by measuring $Y_{X/S}$. To investigate the induced growth repression in the X-press strain without metabolic burden from recombinant product formation, we performed a cultivation without an exogenous plasmid and solely inducing Gp2 expression by addition of L-arabinose. After induction, $Y_{X/S}$ was reduced by half from 0.48 in the uninduced state to levels between 0.24 and 0.27, remaining almost constant throughout the cultivation (Figure 1A). We assumed that any additional reduction of $Y_{X/S}$ is caused by the metabolic burden of heterologous gene expression. During production of SpA in cultivation 25/0.13, the reduction of $Y_{X/S}$ was similar to the "basal" reduction by Gp2 expression, thus the metabolic load of SpA expression had little effect on growth. An additional reduction was observed at higher temperature and $q_{S,0}$. In both cultivations 30/0.25 and 35/0.13, $Y_{X/S}$ decreased throughout the cultivation to values between 0.03 and 0.1. Hence, the metabolic load of recombinant product expression still affected growth of the X-press strain, but it was largely mitigated by induced growth repression. Contrarily, in the reference strain BL21(DE3), $Y_{X/S}$ varied greatly between different cultivation conditions during SpA production (Figure 1B). It decreased at higher induction temperature, so that at 25°C, biomass yield of BL21(DE3) was least affected, while at 35°C, growth was fully arrested. This behavior might stem from an increase in target gene transcript levels competing with host mRNA at elevated temperatures (C. S. Shin, Hong, Bae, & Lee, 1997; Vind, Sorensen, Rasmussen, & Pedersen, 1993).

Impact of process parameters on productivity

At low temperature and $q_{S,0}$ (25/0.13), biomass specific, soluble SpA titer after 12 h was lowest in both strains at 123±4 and 113±7 mg/g in X-press and BL21(DE3), respectively (Figure 2). Raising the temperature at low $q_{S,0}$ from 25 to 35°C drove SpA expression, so that the total titer after 12 h increased to 314±6 and 240±9 mg/g in X-press and BL21(DE3), respectively. It has been shown that the overall protein synthesis rate as well as plasmid replication are dependent on temperature (Farewell & Neidhardt, 1998; Hoffmann & Rinas, 2001). In our experiments, 35°C induction temperature might have resulted in a higher plasmid copy number and concomitant high levels of target gene transcripts, competing for ribosomes with native mRNA, thus increasing recombinant protein expression and decreasing the growth rate. At 25°C, this reaction was possibly shifted in favor of host mRNA due to lower levels of plasmids, resulting in low productivity and little metabolic burden. The highest specific SpA titer was achieved in cultivation 30/0.25, with 387±12 and 351±17 mg/g in X-press and BL21(DE3), respectively, which was expected, since more carbon was available for product formation. However, yield reduction in BL21(DE3) at these process conditions was less than at 35°C, indicating that a decrease in growth rate was not only mediated by foreign protein content, but as hypothesized, by the underlying temperature-dependent mechanisms at transcript level.

An advantage of induced, growth decoupled protein production in the X-press strain is enhanced resource allocation towards recombinant protein. Although in this study, total soluble specific titers did not improve as much as with previously reported products (Lemmerer et al., 2019; Stargardt et al., 2020), specific SpA titers were up to 30% higher in the X-press strain at the end of cultivation compared to the reference strain.

Impact of process parameters on lysis and leakiness

No lysis was detected under any condition during SpA production with BL21(DE3). The X-press strain did not lyse at low$q_{S,0}$, however, in cultivation 30/0.25, lysis increased towards the end of fermentation, so that 7% of cells were lysed after 12 h (Figure 3). Thus, in the later stages of this cultivation, the amount of leaked protein is biased by product release by lysis. Nonetheless, the X-press strain showed higher overall leakiness in response to increased temperature and$q_{S,0}$ compared to BL21(DE3). In cultivation 25/0.13, leakiness reached up to 29% in X-press (Figure 2A), while the reference strain leaked no product at all (Figure 2B). Solely increasing the temperature to 35°C greatly enhanced OM permeability in both strains, so that after 12 h, 82% and 55% of SpA were leaked to the supernatant in X-press and BL21(DE3), respectively. Simultaneously increasing temperature and $q_{S,0}$ (cultivation 30/0.25) led to high leakiness in both strains as well. Up to 90% and 56% of SpA were released to the medium in X-press and BL21(DE3), respectively. Interestingly, the combined effect of temperature and$q_{S,0}$ on leakiness in the reference strain was the same as solely increasing the temperature. In the X-press strain, on the other hand, OM permeability was more sensitive to the simultaneous increase in temperature and $q_{S,0}$. This manifested in much faster product release in cultivation 30/0.25 than in the other cultivations (Figure 2). However, cell lysis commenced after 8 h (Figure 3), which is likely due to high stress caused by the high product formation rate.

From the results obtained in the SpA fermentations we deducted different approaches to extracellular production in the X-press strain: (1) Low$q_{S,0}$ and high temperature are beneficial for maintaining a viable culture and boosting productivity and leakiness over extended fermentation times; (2) moderately increasing temperature and $q_{S,0}$ rapidly enhances leakiness and productivity, but high viability might not be sustained for long fermentation times. Either scenario would allow for efficient capture of the product from the culture supernatant for simplified downstream processing. Controlling leakiness via temperature and$q_{S,0}$ is also possible in the reference strain BL21(DE3). However, these process parameters have a grave impact on productivity as well, thus product location cannot be uncoupled from productivity. For BL21(DE3), this is a double-edged sword: increasing temperature and specific glucose uptake rate greatly enhanced SpA titer, but the cells did not leak more than 60% of product to the medium. Hence, capturing the target protein from the cells or from the medium, respectively, would result in large product losses in both scenarios.

Table 1 shows an overview of previous studies on the effect of cultivation temperature and q_S on leakiness in different *E. coli* hosts, that have not been engineered for the purpose of protein secretion. There is no fully consistent behavior among different *E. coli* strains. In fact, differences exist even between similar strains. Reasons for this might be the used promoter system or product and resulting differences in the energy requirement, resource handling within the cell and, ultimately, membrane structure and properties. Other environmental factors, like medium composition and aeration, were also shown to affect OM permeability (Orr et al., 2012; Ukkonen, Veijola, Vasala, & Neubauer, 2013), which makes a direct comparison between different studies even harder. Our results agree with most studies listed in Table 1 that showed a positive correlation between leakiness and both temperature and q_S . This might be due to a change in fatty acid composition and therefore rigidity of the cell envelope (Arneborg, Salskov-Iversen, & Mathiasen, 1993; Shokri et al., 2002) or a change in OM proteins that might influence the transport to the medium (Bäcklund et al., 2008). However, given the inconsistent results in literature, more rigorous examination of the relationship between expression system, process parameters, like temperature and q_S , membrane properties and resulting leakiness is needed to gain more mechanistic understanding, not only for the strains used in this study, but for all future research on this topic.

In leaky mutants, the increased secretion across the OM is most often due to mutations in genes related to membrane proteins, lipopolysaccharides or the peptidoglycan layer (Z. Y. Chen et al., 2014; Müller, Wetzel, Flaschel, Friehs, & Risse, 2016; Orr et al., 2012; H. D. Shin & Chen, 2008; Zhou et al., 2018). These genes were not manipulated during the construction of the X-press strain. Thus, the question is raised, how Gp2 expression can have an impact on membrane properties. Clearly, inhibiting the host RNA-polymerase, a most central enzyme in cell proliferation, can disturb practically any metabolic pathway. So far, the chain of causality between Gp2 expression and increased membrane permeability remains obscure. Currently, we are investigating the effects of Gp2 at the transcriptome and proteome level.

Production of VHH in BL21(DE3) and X-press

The cultivation conditions that resulted in the highest productivity of SpA in each strain (30/0.25) were repeated with the second model protein, VHH, and fermentations were assessed after 14 h induction time. The results are summarized in Table 2. The biomass growth in both strains was less affected compared to the corresponding SpA cultivations. In the X-press strain, the biomass yield reduction was close to the "basal" growth repression by Gp2 induction. In BL21(DE3), biomass yield was reduced by less than 0.1 g/g. This was likely due to the much lower amount of produced recombinant product compared to SpA and, as a result, a lower metabolic burden (Bentley et al., 1990; Bienick et al., 2014). Total productivity of soluble VHH was greatly enhanced in the X-press strain compared to the reference strain. Although inclusion body formation was detected in both strains (Supporting Information 4), the induced growth repression and enhanced secretion ability of the X-press strain seemed to have a beneficial effect on solubility of VHH, which is difficult to fold due to its disulfide bridges (de Marco, 2009; Liu & Huang, 2018). Also the amount of secreted protein was greatly improved in the X-press strain and was comparable to the SpA cultivations, although lysis was negligible during VHH production. Overall, the cultivations with the second model protein confirmed that the selected settings of process parameters ($T = 30$°C, $q_{S,0} = 0.25$ g/g/h) lead to efficient product secretion in the X-press strain, while product location in BL21(DE3) is inefficiently partitioned both inside and outside the cell. The issue of insoluble product aggregation might be addressed in further development, for instance by inducer titration or similar approaches, to fine tune expression levels and thus further enhance soluble productivity.

Conclusion

We could demonstrate the applicability of the novel *E. coli*X-press strain for extracellular production of recombinant proteins. We narrowed down the design space, in which extracellular protein production is favored without sacrificing viability: cultivation temperatures between 30 and 35°C and$q_{S,0}$ between 0.13 and 0.25 g/g/h enhanced both leakiness and productivity while keeping cell lysis to a minimum. The process

parameters both individually and interactively affected total product titer and leakiness in a positive manner in both investigated expression hosts. By inducible growth repression, the novel *E. coli* X-press strain showed less susceptibility to the metabolic burden of recombinant protein production and thus allows for tighter process control due to reduced variability across different process conditions. Lastly, we showed that the X-press strain can achieve high titers of different classes of recombinant protein and leaks up to 90% of all soluble product. Therefore, this strain is a promising candidate for extracellular protein production in current fed-batch applications or for future continuous manufacturing. Further research should be directed towards the relationship between different expression systems, process parameters and their implications on periplasmic protein release.

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Figure 1 Biomass yield in cultivations of X-press (A) and BL21(DE3) (B) producing SpA.

Figure 2 Intra- and extracellular soluble SpA titer in cultivations of X-press (A) and BL21(DE3) (B). Annotations above the columns represent leakiness in percent.

Figure 3 Cell lysis in cultivations of the X-press strain producing SpA.