Inhibition of E. coli Host RNA Polymerase Allows Efficient Extracellular Recombinant Protein Production by Enhancing Outer Membrane Leakiness

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With the growing interest in continuous cultivation of Escherichia coli, secretion of product to the medium is not only a benefit, but a necessity in future bioprocessing. In this study, it is shown that induced decoupling of growth and heterologous gene expression in the E. coli X-press strain (derived from BL21(DE3)) facilitates extracellular recombinant protein production. The effect of the process parameters temperature and specific glucose consumption rate ($q_g$) on growth, productivity, lysis and leakiness, is investigated, to find the parameter space allowing extracellular protein production. Two model proteins are used, Protein A (SpA) and a heavy-chain single-domain antibody (VHH), and performance is compared to the industrial standard strain BL21(DE3). It is shown that inducible growth repression in the X-press strain greatly mitigates the effect of metabolic burden under different process conditions. Furthermore, temperature and $q_g$ are used to control productivity and leakiness. In the X-press strain, extracellular SpA and VHH titer reach up to 349 and 19.6 mg g$^{-1}$, respectively, comprising up to 90% of the total soluble product, while keeping cell lysis at a minimum. The findings demonstrate that the X-press strain constitutes a valuable host for extracellular production of recombinant protein with E. coli.

1. Introduction

E. 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.\[1-3\] However, the product is usually expressed inside the cell, which requires cell disruption in downstream processing, leading to the release of unwanted host cell proteins and other contaminants, like lipids and DNA.\[4\] If the target protein is produced as insoluble inclusion bodies (IBs), additional IB processing is needed. Especially with the growing interest in continuous manufacturing,\[5,6\] 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.\[7\] 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.\[1\] Numerous studies on how to increase leakiness during cultivation exist and several reviews cover this research in detail.\[1,3,8\]

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.\[1\] Another approach is the generation of leaky E. coli mutants. Many expression systems that show permanently high leakiness have been engineered to date. Their OM structure is usually altered by mutations in cell envelope genes and signal peptides are optimized for higher translocation efficiency.\[1,3\] However, detailed process information at bioreactor scale is often missing for these strains.

Another reported strategy is the enhancement of OM permeability via cultivation conditions. In this regard, previous research focused mainly on the process parameters temperature
and specific growth rate ($\mu$). Shokri, Sanden, and Larsson[10] showed that growth rate-dependent changes in the membrane composition affect protein leakage. In their study, in continuous cultivation of E. coli W3110, leakage had an optimum at a dilution rate of 0.3 h$^{-1}$ and declined upon lowering or increasing $\mu$. Similar results were obtained in fed-batch studies of W3110[11] and a K12 derivative[12] in which an increase in $\mu$ led to enhanced leakage. Contrarily, Rinna and Hoffmann[13] stated that $\mu$ 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 that $\mu$ and leakage are inversely correlated.[14] Adverse reports can also be found about the influence of temperature on OM leakage for different E. coli strains. While Rodriguez-Carmona et al.[15] found that leakage of a Fab was enhanced at lower temperatures, several other studies suggest that increased temperature drives periplasmic protein release.[13,14,16] Controlling leakage via temperature and $\mu$ is an interesting approach since it does not require alteration of the chemical environment and is easy to implement. Thus, these process parameters are frequently investigated during upstream process development.[14,17–19] However, the contrary results in the aforementioned studies illustrate that the effects that temperature and $\mu$ have on OM leakage 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 consumption rate ($q_g$, linked to $\mu$ via the biomass yield $Y_{X/S}$) on OM leakage of an E. coli X-press. This strain is a proprietary expression technology recently developed by enGenes Biotech GmbH.[20,21] 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.

2. Experimental Section

2.1. Strains

Two E. coli strains were used in this study: the X-press strain, a BL21(DE3) derivate patented by enGenes Biotech GmbH[20,21] 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 knock-out of the araABCD operon. Details about the genotype can be found in ref.[21] For recombinant protein production, both strains were transformed with a pET30a plasmid,[21] carrying the gene coding for 1) the IgG-binding domains of Protein A from S. aureus (SpA) with the pelB signal sequence or 2) an anti-TNFRI VH4 (DOM101) with the ompA signal sequence.[21] Both proteins were His-tagged at the C-terminus. Protein sequences are listed in Table S1, Supporting Information 1.

2.2. Media

The semi-defined medium for the pre-culture was prepared according to re.[24] to produce 3 g L$^{-1}$ cell dry weight. For bioreactor cultivations, defined minimal media[25] was used, with glucose as carbon source. The initial glucose concentration was 20 g L$^{-1}$ and the substrate feed had a glucose concentration of 400 g L$^{-1}$.

2.3. 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 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$^{-1}$. The batch volume was 1 L. The culture broth was supplied with a mixture of air and pure oxygen at 10 L min$^{-1}$ 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 the addition of NH$_4$OH (12.5%). The temperature was controlled with a heating blanket and cooling finger and kept at 37 °C, except during induction (described below).

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), the substrate was fed to reach a cell dry weight concentration of 30 g L$^{-1}$. Subsequently, expression of SpA or VH4 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.
2.4. Design of Experiments

The effect of temperature and $q_S$ on growth, productivity, lysis, and leakiness during SpA production was studied in a DoE. Since the growth of the X-press strain is repressed by Gp2 expression during induction, the authors chose to apply a constant substrate feed rate in the experiments. In the DoE, this was reflected in the first factor as $q_S$ with respect to biomass at the start of induction ($q_{S,0}$). This parameter can thus be scaled to different biomass concentrations and reactor volumes. First, a full factorial screening DoE was performed using both E. coli strains with $q_{S,0}$ set to 0.13, 0.25, and 0.50 g g$^{-1}$ h$^{-1}$, respectively. The temperature during induction, the second factor, was 25, 30, or 35 °C, respectively (Figure 1). The different parameter settings are hereafter referred to as “temperature (°C)/$q_{S,0}$ (g g$^{-1}$ h$^{-1}$)”, for example, 30/0.25. At $q_{S,0} = 0.5$ g g$^{-1}$ h$^{-1}$, the physiological capabilities of the X-press strain were far exceeded (manifested in glucose accumulation and lysis, see Figures S1–S2, Supporting Information) and linear regression did not result in significant model coefficients, thus it was decided to omit the data of these experiments from further analysis in this study. After the screening DoE, the X-press strain was further characterized in a refined DoE comprising additional experiments in the design space (Figure 1). Processes were analyzed as described below and supplementary time-resolved data of the DoE comprising cell dry weight concentration, glucose concentration, $\mu$, specific product formation rate, and carbon balances are given in Figures S3–S12, Supporting Information. Finally, the process conditions leading to the highest productivity during SpA cultivations were applied on a strain carrying a plasmid encoding VHH and evaluated after 14 h of induction.

2.5. 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 72 h. Dry biomass concentration was then determined by weighing the dry pellets. Concentrations of residual glucose and t-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$_3$PO$_4$ and the flow rate constant at 0.5 mL min$^{-1}$.

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

$$Y_{X/S,0} = \frac{m_{X,i} - m_{X,0}}{m_{S,i}}$$

$$Y_{X/S,n} = \frac{m_{X,n} - m_{X,i}}{m_{S,n} - m_{S,i}}$$

$Y_{X/S,0}$ is the biomass yield before induction (g g$^{-1}$); $m_{X,i}$ is the biomass at the time of induction (g); $m_{X,0}$ is the biomass at the time of inoculation (g); $m_{S,i}$ is the consumed sugar at the time of induction (g); $Y_{X/S,n}$ is the biomass yield after induction at time point $n$ (g g$^{-1}$); $m_{X,n}$ is the biomass after induction at time point $n$ (g); $m_{S,n}$ is the consumed sugar after induction at time point $n$ (g).

2.6. 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. For the calculations, a cellular DNA content of 31 mg g$^{-1}$ was assumed, which was taken from the literature. 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 Figure S13 and Equation S1, Supporting Information. The amount of lysed cells was calculated according to Klein et al. and expressed as a percent of total biomass concentration as shown in Equation (3):

$$\text{lysed cells} = \frac{x_1}{x_1 + x} \times 100$$

$x_1$, amount of lysed biomass (g L$^{-1}$); $x$, cell dry weight (g L$^{-1}$).

2.7. Product Analysis

For SpA quantification, 10 mL culture were centrifuged for 10 min 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 min (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 Polyphenyl; 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.

IB 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 min (10 000 rcf, 4°C). 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 min (10 000 rcf, 4°C). Subsequently, the pellet was resuspended in 1 mL ultrapure water, diluted with 1.5× Laemmli buffer. A VHH standard (5 g L\(^{-1}\)) was diluted in 2× Laemmli buffer. The samples and standard were then incubated at 95 °C for 15 min. 10 μL of sample and 5 μL of the standard were loaded onto precast SDS gels (8-16%, Mini-PROTEAN Tetra-Cell, PDV, Sweden) and were run at 180 V for 30 min 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). SDS-PAGE analysis of SpA in the culture supernatant of E. coli X-press and the soluble fraction of the cell lysate of E. coli BL21(DE3) was done analogously, with the exception that 2× Laemmli buffer was used.

2.8. Calculation of Leakiness

The quotient of the soluble extracellular and total soluble product (leakiness) in per cent was calculated using Equation (4):

\[
\text{leakiness} = \frac{w_{\text{pex}}}{w_{\text{pex}} + w_{\text{pin}}} \times 100
\]  

(4)

\(w_{\text{pex}}\): biomass specific intracellular product concentration (mg g\(^{-1}\)); \(w_{\text{pex}}\): biomass specific extracellular product concentration (mg g\(^{-1}\)).

2.9. Modelling

The data from the refined DoE (all SpA cultivations with E. coli X-press except \(q_{S,0} = 0.5\)) at 12 h after induction were used to generate a linear model describing \(Y_{X/S}\), total biomass specific SpA titer and leakiness as functions of temperature and \(q_{S,0}\) by multiple linear regression. The software MODDE 10 (Umetrics, Umeå, Sweden) was used.

3. Results and Discussion

The process parameters temperature and \(\mu\) are important factors in bioprocess development and are known to have an impact on the leakiness of different E. coli strains (Table 1), although conclusive understanding is still missing. Stargardt et al.\(^{[21]}\) previously reported that the E. coli X-press strain tends to leak periplasmic proteins to the supernatant. In this follow-up study, we performed further characterization of the X-press strain in fed-batch cultivations by investigating the influence of temperature and \(q_{S,0}\) on leakiness. In this study, we used some of the studies to determine the substrate feed rate as parameters. For consistency, these parameters were summarized as \(q_{S,0}\) here. \(\leftrightarrow\): positive correlation; \(\rightarrow\): negative correlation; \(\leftrightarrow\): leakiness increased, then decreased with parameter; \(\rightarrow\) parental strain: E. coli K12; \(\rightarrow\) parental strain: E. coli BL21(DE3).

### Table 1. Studies investigating the effect of temperature or \(q_{S,0}\) on leakiness in different E. coli strains.

| Strain          | Inducer | Parameter\(^{1}\) | Effect on leakiness\(^{2}\) of | Reference |
|-----------------|---------|-------------------|--------------------------------|-----------|
| TG\(^{1}\)      | heat    | \(T/q_{S}\)       | +                              | [13]      |
| HMS174(DE3)\(^{3}\) | IPTG    | \(T\)             | –                              | [15]      |
| O17f\(^{1}\)    | Constitutive | \(q_{S}\) | +                        | [12]      |
| W3110f\(^{1}\) | IPTG    | \(q_{S}\)         | +                              | [12]      |
| W3110f\(^{1}\) | Ampicillin | \(q_{S}\) | +\(\rightarrow\)/ – \(\leftrightarrow\) | [10]      |
| W3110f\(^{1}\) | IPTG    | \(q_{S}\)         | +                              | [13]      |
| C41(DE3)\(^{4}\) | arabinose | \(T/q_{S}\) | –                              | [14]      |
| BL21(DE3)\(^{5}\) | IPTG/lactose | \(T\) | +\(\rightarrow\)/ – \(\leftrightarrow\) | [29]      |
| BL21(DE3)\(^{5}\) | IPTG    | \(T\)             | +\(\rightarrow\)/ – \(\leftrightarrow\) | This study |
| X-press\(^{5}\) | IPTG    | \(T/q_{S}\)       | +                              | This study |

\(^{1}\) Some of the studies used \(\mu\) or the substrate feed rate as parameters. For consistency, these parameters are summarized as \(q_{S,0}\) here; \(\leftrightarrow\): positive correlation; \(\rightarrow\): negative correlation; \(\leftrightarrow\): leakiness increased, then decreased with parameter; \(^{2}\) parental strain: E. coli K12; \(^{3}\) parental strain: E. coli BL21(DE3).

3.1. Screening of Parameter Space and Comparison between X-Press and BL21(DE3)

3.1.1. 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 a metabolic burden.\(^{[30]}\) This burden arises from limitations in resource allocation between vegetative biomass and recombinant protein and is often associated with a decrease in growth rate and ultimately cell lysis.\(^{[31,32]}\) In the X-press strain, the metabolic burden may be reduced by decoupling cell proliferation from recombinant protein production. It was shown that Gp2 expression alone, independent of plasmid-based protein expression, reduces \(Y_{X/S}\) in fed-batch cultivation up to 55%\(^{[21]}\) and therefore mitigates the effect of recombinant protein production on growth. In our study, we assessed the impact of the selected process parameters on growth by measuring \(Y_{X/S}\). In the X-press strain, \(Y_{X/S}\) was reduced by half from 0.39 in the uninduced state.
to levels between 0.17 and 0.20 during the production of SpA in cultivation 25/0.13 (Figure 2A). Under the same conditions, growth of BL21(DE3) was less affected and $Y_{X/S}$ was only reduced from 0.38 to 0.28. Reduced growth in the X-press strain compared to BL21(DE3) can be attributed to growth repression by Gp2. $Y_{X/S}$ was further reduced in both strains with increasing temperature and $q_{S,0}$ to 30/0.25 (Figure 2B), as well as only increasing temperature to 35/0.13 (Figure 2C). In BL21(DE3), this is probably caused by the higher metabolic load of SpA expression, while elevated Gp2 expression in the X-press strain might additionally have decreased $Y_{X/S}$. The effect of different cultivation conditions on growth was much stronger in BL21(DE3), with distinct temperature dependence. This behaviour might stem from an increase in target gene transcript levels competing with host mRNA at elevated temperatures \[^{31,34}\]. Contrarily, repressing host RNA polymerase activity in the X-press strain greatly mitigated the effect of different parameter settings on growth.

### 3.1.2. 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\(^{-1}\) in X-press and BL21(DE3), respectively (Figure 2D). 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\(^{-1}\) in X-press and BL21(DE3), respectively (Figure 2F). It has been shown that the overall protein synthesis rate, as well as plasmid replication, are dependent on temperature. \[^{35,36}\] 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 (Figure 2E), with 387 ± 12 and 351 ± 17 mg g\(^{-1}\) 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 the transcript level.

An advantage of induced, growth decoupled protein production in the X-press strain is enhanced resource allocation toward recombinant protein. Although in this study, total soluble specific titers did not improve as much as with previously reported products, \[^{21,22}\] specific SpA titers were up to 30% higher in the X-press strain at the end of cultivation compared to the reference strain.

### 3.1.3. 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 toward the end of fermentation so that 7% of cells were lysed after 12 h (Figure 2G). 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 29% in X-press, while the reference strain leaked no product at all (Figure 2D). 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 (Figure 2F). Simultaneously increasing temperature and $q_{S,0}$ (cultivation 30/0.25) led to high leakiness in both strains as well (Figure 2E). 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 $q_{S,0}$ 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. However, cell lysis commenced after 8 h (Figure 2G), which is likely due to the high stress caused by the high product formation rate.

From the results obtained in the screening DoE, 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 overextended fermentation times; 2) moderating 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. The advantageous purity of the product in the culture supernatant in comparison with cell lysate was demonstrated by SDS-PAGE analysis (Figure 2H). Protein in the supernatant showed similar purity in both strains. 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 $q_{S,0}$ 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 the medium, respectively, would result in large product losses in both scenarios.

### 3.2. Refined Design of Experiments for E. coli X-press

The X-press strain was further characterized to 1) find the parameter space in which leakiness is most sensitive to changes in temperature and $q_{S,0}$ and 2) to optimize these process parameters for extracellular protein production. For this, additional cultivation conditions were tested in narrower design space (Figure 1). The rationale for limiting the temperature below 35 °C at elevated $q_{S,0}$ was to avoid high stress, since we observed lysis at cultivation 30/0.25 in the screening DoE. Similarly, $q_{S,0}$ was not increased further than 0.25 due to the observed physiological limitations in cultivation 30/0.25 and at initially tested higher $q_{S,0}$ (see section 2.4). A linear model was fit to
Figure 2. Results from cultivations of X-press and BL21(DE3) producing SpA within the screening DoE: $Y_{X/S}$ in cultivations A) 25/0.13, B) 30/0.25, and C) 35/0.13. Intra- and extracellular soluble SpA titer in cultivations D) 25/0.13, E) 30/0.25, and F) 35/0.13. Annotations above the columns represent leakiness in percentage. G) Cell lysis in cultivations of X-press. Shown data are the mean of 3 technical replicates ± SD. H) SDS-PAGE analysis of SpA from the cell lysate of BL21(DE3) (Lane 1), unprocessed culture supernatant of X-press (Lane 2), and unprocessed culture supernatant of BL21(DE3) (Lane 3). All three samples were collected after 12 h of induction at conditions 30/0.25.
describe $Y_{X/S}$, total soluble titer and leakiness as functions of the process parameters. The time-resolved data from these additional cultivations for $Y_{X/S}$, total soluble titer, leakiness and lysis are included in Figures S14–S16, Supporting Information 5. The model coefficients and fit summaries are listed in Figure S17 and Table S2, Supporting Information 6. Overall, the results from the refined DoE underline the observations from the screening DoE. As shown in Figure 3A, $Y_{X/S}$ is mainly influenced by temperature in the X-press strain. Titer, on the other hand, increases both with temperature and $q_{S,0}$ (Figure 3B), owing to higher expression rates and substrate flux. However, above 30 °C the effect of temperature on titer decreases, indicating that SpA expression rates are close to their maximum at that temperature. Leakiness in the X-press strain also increases with both process parameters (Figure 3C). Notably, OM permeability in this strain is easily triggered, so that small increases in temperature and $q_{S,0}$ at relatively low levels have a great influence on leakiness and a large part of the parameter space allows for more than 80% of the periplasmic product to be released to the medium. Like the titer, the effect of temperature on leakiness slightly diminishes above 30 °C. Overall, extracellular protein production with E. coli X-press is most favorable at elevated temperatures and $q_{S,0}$.

However, the operating range is limited by the physiological capacities of the cell. More than 6% lysis was observed in the late stages of cultivation 30/0.25, all other conditions led to lysis of only below 2% (Figure 3D). Thus, $q_{S,0}$ should be limited to maximally 0.25 g g$^{-1}$ h$^{-1}$, especially at temperatures of 30 °C and above.

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 protein secretion. There is no fully consistent behaviour among different E. coli strains. 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, might also affect OM permeability, 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 or a change in OM proteins that might influence the transport to the medium.
However, given the inconsistent results in the 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 mostly due to mutations in genes related to membrane proteins, lipopolysaccharides or the peptidoglycan layer.\[9,37,40–42\] 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. Inhibiting the host RNA-polymerase, a most central enzyme in cell proliferation, can disturb practically any metabolic pathway. While the focus of the present study was the investigation of the process behaviour of the X-press strain, the molecular effects of Gp2 expression on the membrane will be explored on the transcriptome and proteome level in future research.

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

As a proof of concept for extracellular protein production of different product classes, 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. This was likely due to the much lower amount of produced recombinant product compared to SpA and, as a result, a lower metabolic burden.\[31,32\] Total productivity of soluble VHH was greatly enhanced in the X-press strain compared to the reference strain. Although IB formation was detected in both strains (Figures S18 and S19, Supporting Information 7), the induced growth repression and enhanced secretion ability of the X-press strain seemed to have a beneficial effect on the solubility of VHH, which is difficult to fold due to its disulfide bridges.\[43,44\] 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. Although the chosen cultivation conditions led to high leakiness in the X-press strain, lower temperatures may be required to reduce IB formation and improve soluble VHH productivity. This shows that different proteins require further screening of the design space to find the optimal conditions for high soluble expression of each product while keeping leakiness high. Still, 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$^{-1}$ h$^{-1}$) 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 further addressed by inducer titration to fine-tune expression levels and thus further enhance soluble productivity.

4. Conclusion

We could demonstrate the applicability of the novel $E. coli$ X-press strain for extracellular production of recombinant proteins. We explored the design space, in which extracellular protein production is favored without sacrificing viability: For the soluble model protein SpA, cultivation temperatures between 30 and 35 °C and $q_{s,0}$ of up to 0.25 g g$^{-1}$ h$^{-1}$ enhanced both leakiness and productivity while keeping cell lysis to a minimum. The process parameters affected total product titer and leakiness positively 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, although temperature dependence of growth was still observed. Finally, 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 future continuous manufacturing. However, additional screening of the design space for different products may be required, specifically for proteins that are prone to IB formation. Further research should be directed toward the relationship between different expression systems, process parameters, and their implications on periplasmic protein release.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords**

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