

Triggering outer membrane leakiness of a novel *E. coli* strain for recombinant protein production

Jens Kastenhofer

Technische Universität Wien

Lukas Rettenbacher

Technische Universität Wien

Lukas Feuchtenhofer

engenes Biotech GmbH

Jürgen Mairhofer

engenes Biotech GmbH

Oliver Spadiut (✉ oliver.spadiut@tuwien.ac.at)

TU Wien <https://orcid.org/0000-0003-0916-0644>

Research

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Abstract

Background

Recombinant proteins in *Escherichia coli* are 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 a novel *E. coli* production host for growth decoupled recombinant protein production that can leak up to 90% of recombinant protein to the extracellular space. We investigated the effects of the process parameters temperature and specific glucose uptake rate on physiology, productivity, lysis and leakiness. Two model proteins were used, Protein A and a VHH single-domain antibody, and performance was compared to the industrial standard strain BL21(DE3).

Results

We show that inducible growth repression in the novel *E. coli* strain enGenes-X-press, the effect of the metabolic burden on host physiology can be greatly reduced compared to BL21(DE3). Furthermore, in both strains, increasing temperature and specific substrate enhanced productivity and leakiness. Using the enGenes-X-press strain, extracellular Protein A and VHH titer reached up to 349 mg/g and 19.6 mg/g, respectively, comprising between 80 and 90% of total soluble product, while keeping cell lysis to a minimum. BL21(DE3) leaked 198 mg/g and 3.9 mg/g of Protein A and VHH to the medium, accounting for only 56% and 34% of total soluble product, respectively.

Conclusions

We confined the parameter space in which outer membrane leakiness can be controlled, while maintaining cell viability. Moreover, our findings demonstrate that the enGenes-X-press strain constitutes a superior host for extracellular production of recombinant protein.

Background

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 [1–3]. In the biopharmaceutical sector in 2018, it produced 22% of all therapeutic proteins with active licenses in the USA and the EU [4]. Although this marked a decrease in the share of microbials on the biotherapeutic market compared to the decades before, the emergence of biologics, like antibody fragments (Fab), single domain antibodies (sdAb) and other scaffolds, which can be expressed in prokaryotes, leads to a resurgence of *E. coli* as a production host [5–8].

The growing number of products and biosimilars in the field puts pressure on industry regarding manufacturing costs, time-to-market and flexibility. In recent years, the industrial and academic community have propagated a shift from batch-wise to continuous processing to tackle these issues [9–14]. Apart from reducing capital investment due to smaller plant footprints, continuous manufacturing

allows for more flexible change between high- and low-demand products, less down time of unit operations, less process variability and reduced degradation of the target molecule due to less residence time in the reactor [9–11]. Integrated continuous operation has been demonstrated in animal cell [12, 13] and even *E. coli* processes [14]. However, in the latter, the product is typically located 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 [15]. If the target protein is produced as insoluble inclusion bodies (IBs), additional IB processing is needed. Thus, continuous manufacturing in *E. coli* is still hard to realize to date. One strategy to bring *E. coli* to continuous manufacturing is soluble, extracellular protein production.

Secretion of recombinant protein to the medium furthermore enhances solubility, stability and biological activity of the product [16]. 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 [3]. Numerous studies on how to increase leakiness during cultivation exist and several reviews cover this research in detail [2, 3, 17].

One approach to increase leakiness is chemical permeabilization by addition of media supplements, like Triton-X, Tween or EDTA. However, those additives usually have detrimental effects on the viability of the cells and might harm the product [3]. 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 [3, 18, 19]. Wacker Biotech GmbH developed a commercially available strain that can secrete several grams of different products per liter [20, 21]. However, information on process design is not available for this proprietary strain.

Different process parameters during cultivation, like temperature, specific growth rate, pH and aeration, have been proposed to have an effect on the fluidity, composition and therefore leakiness of the OM [22]. Especially temperature and growth rate were shown to have a significant effect on leakiness during cultivation [23–28]. Strategies based on these parameters are easy to implement, yet consistent understanding of the (possibly combined) effect of temperature and growth rate on product release, physiology and productivity is still missing.

In this study, we investigated the influence of the process parameters cultivation temperature and specific glucose uptake rate on OM leakiness of a novel *E. coli* expression host. The X-press strain is a proprietary expression technology developed by enGenes Biotech GmbH [29]. It 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 [29, 30]. In previous, unpublished experiments, the X-press strain showed high tendency to leak periplasmic protein to the medium. Therefore, in the present research, we further investigated its response to the process parameters temperature and specific substrate uptake rate in fed-batch cultivations under the controlled conditions of a benchtop bioreactor. 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 single domain antibody (VHH). The processes were analyzed with respect to physiology, 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 possible application in continuous manufacturing.

Results And Discussion

In this study, we investigated the influence of temperature and specific glucose uptake rate on physiology, productivity, lysis and leakiness of two different *E. coli* chassis strains. These process parameters are important factors in bioprocess development and have been shown to have an impact on leakiness before [23–28]. Thus, understanding the impact of temperature and specific glucose uptake rate on OM permeability is necessary for the successful control of product location. As a benchmark strain we chose *E. coli* BL21(DE3), since it is the most widely used *E. coli* strain for recombinant protein production due to its fast growth, low acetate production, diminished protease content and its powerful T7 expression system [31, 32]. The responses of physiology, productivity, lysis and leakiness to three combinations of cultivation temperature and $q_{S,0}$ (30/0.25, 25/0.13, 35/0.13) were first studied 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 SpA Production In BL21(DE3)

It has been long known that heterologous expression in plasmid-based *E. coli* systems has a grave impact on cell physiology, widely known as *metabolic burden* [33]. This burden is often associated with a decrease in growth rate or, ultimately, cell lysis [34, 35]. We assessed the impact of the selected process parameters on cell physiology by measuring the biomass yield, $Y_{X/S}$ and lysis. Yield reduction varied greatly between different cultivation conditions (Fig. 1). It increased with induction temperature, so that at 25 °C, biomass yield of BL21(DE3) was least affected, while at 35 °C, growth was fully arrested. We hypothesize, that this behavior stems from an increase in target gene transcript levels competing with host mRNA at elevated temperatures, which might also be reflected in SpA productivity.

Indeed, at low temperature and $q_{S,0}$ (25/0.13), biomass specific, soluble SpA titer was lowest at 113 ± 7 mg/g (Fig. 2A) after 12 h. Raising the temperature at low $q_{S,0}$ from 25 to 35 °C drove SpA expression, so that the total titer after 12 h more than doubled to 240 ± 9 mg/g. It has been shown that the overall protein synthesis rate as well as plasmid replication are dependent on temperature [36, 37]. In our experiment, 35 °C induction temperature might have resulted in 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 growth rate. At 25 °C, this reaction was possibly shifted in favor of host mRNA due to lower levels of plasmids, which could explain low productivity and little metabolic burden. The highest specific SpA titer was 351 ± 17 mg/g 12 h after induction in cultivation 30/0.25, which was expected since more carbon was available for product formation. However, yield reduction 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.

No lysis was detected at all conditions during SpA production with BL21(DE3). Leakiness in the reference strain reached 50–60% in both cultivations 30/0.25 and 35/0.13 after 12 h, accounting for 198 ± 9 mg/g and 133 ± 3 mg/g extracellular SpA titer, respectively. However, protein secretion commenced only between 4 and 8 hours (Fig. 2). In cultivation 25/0.13, no product at all was leaked to the medium. Those results are congruent with previous findings, that state OM permeability increases with higher temperature and growth rates, respectively [23–27]. The similarity in leakiness between conditions 30/0.25 and 35/0.13 indicate a combined effect of temperature and substrate uptake rate: increasing temperature by 5 °C and feed rate by 0.12 g/g/h had the same effect on leakiness as an increase of 10 °C with no change in feed rate.

These results demonstrate, that controlling leakiness via temperature and specific substrate uptake rate is possible for BL21(DE3) within the investigated parameter space. 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 and therefore BL21(DE3) does not constitute an effective host for extracellular protein production.

Impact of process parameters on SpA production in enGenes X-press

By uncoupling growth from recombinant protein production via co-expression of Gp2, the novel enGenes X-press production host can achieve high specific product yields and is suitable for expression of toxic proteins [29, 30]. In preliminary studies, we observed that the X-press strain leaked up to 90% of periplasmic protein to the supernatant. In this study, we characterized the strain by investigating the

response of physiology, productivity, lysis and leakiness to changes in temperature and specific substrate uptake rate, in the same design space as the reference strain BL21(DE3).

Additionally, to investigate the growth repression induced by expression of Gp2 without metabolic burden from recombinant product, we cultivated the X-press strain without an exogenous plasmid and solely inducing Gp2 expression by addition of L-arabinose. After induction, the biomass yield was reduced up to half from 0.48 in the uninduced state to levels between 0.24 and 0.27, remaining almost constant throughout the cultivation (Fig. 3). We assumed that any additional reduction in biomass yield is caused by the metabolic burden of heterologous gene expression. During production of SpA in cultivation 25/0.13, the reduction of biomass yield 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, biomass yield 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, so that variability in growth across different cultivation conditions was greatly reduced compared to the BL21(DE3) strain.

The effect of temperature and substrate uptake rate on total soluble SpA productivity of the X-press strain was similar to the BL21(DE3) reference strain. Generally, higher temperature and substrate uptake rate drove SpA production (Fig. 4). At low temperature and $q_{S,0}$, productivity was lowest, while increasing the temperature to 35 °C boosted final productivity more than 2.5-fold, from 123 ± 4 mg/g to 314 ± 6 mg/g after 12 h of induction. As in BL21(DE3), this is likely due to higher protein translation and plasmid replication rates. The highest amount of total soluble SpA, 387 ± 12 mg/g after 12 h, was produced in cultivation 30/0.25, where more carbon was available for product formation.

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 (Fig. 5). 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 than BL21(DE3), though the triggering mechanisms remained similar. Increasing $q_{S,0}$ and temperature individually both triggered leakiness, while simultaneous increase resulted in an amplified effect. Between 80 and 90% of SpA was found in the supernatant after 12 h at all conditions, except in cultivation 25/0.13, which only yielded 29% of extracellular product (Fig. 4). While lysis was low in cultivation 30/0.25, extracellular SpA reached 266 mg/g after 8 h, comprising 81% of total product.

From the results obtained in the SpA fermentations we deduced 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.

Production Of VHH In BL21(DE3) And X-press

The cultivation conditions that resulted in the highest productivity in each strain ($T = 30\text{ }^{\circ}\text{C}$, $q_{S,0} = 0.25\text{ g/g/h}$) were repeated with a second model protein, a VHH single domain antibody, and fermentations were assessed after 14 h induction time. The results are summarized in Table 1. 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 C-mol/C-mol. This was likely due to the much lower amount of produced recombinant product compared to SpA and, as a result, a lower metabolic load [34, 35]. 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 (Additional file 1), 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 more difficult to fold due to its disulfide bridges [8, 38]. 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 ($T = 30\text{ }^{\circ}\text{C}$, $q_{S,0} = 0.25\text{ g/g/h}$) lead to efficient product secretion in the X-press strain, while product location in BL21(DE3) is unefficiently 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.

Table 1
Summary of the cultivations for the production of VHH.

Strain	$Y_{X/S}$ [C-mol/C-mol]	W_{Pin} [mg/g]	W_{Pex} [mg/g]	Leakiness [%]	Lysis [%]
BL21(DE3)	0.37 ± 0.01	7.6 ± 0.6	3.9 ± 0.1	34 ± 2	1 ± 0
X-press	0.22 ± 0.01	3.3 ± 0.4	19.6 ± 0.5	86 ± 2	3 ± 0

Cause Of Enhanced Leakiness In enGenes X-press

A plethora of leaky mutants have been described in literature before, and the increased secretion across the OM is most often due to mutations in genes related to membrane proteins, lipopolysaccharides or the peptidoglycan layer [3, 18, 19]. These genes were not manipulated during the construction of the enGenes X-press host. 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. At the time of preparing this manuscript, the effects of Gp2 at the transcriptome level were being investigated.

Conclusion

In this study, we present an approach to trigger periplasmic protein release in a novel *E. coli* strain solely via cultivation temperature and substrate uptake rate. 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 enhance both leakiness and productivity while keeping 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. The process understanding gained in these fed-batch studies could ultimately be transferred to continuous cultivation, where steady-state would allow for tighter control of leakiness and productivity [39]. By inducible growth repression, the novel expression host enGenes X-press 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 80–90% 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.

Materials And Methods

Strains

Two *E. coli* strains were used in this study: the X-press strain, a BL21(DE3) derivative patented by enGenes Biotech GmbH [29], 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 [40] 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 [41]. Both proteins were His-tagged at the C-terminus. Protein sequences are listed in Additional file 2.

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 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.35 g/L $(\text{NH}_4)_2\text{SO}_4$, 1.11 g/L NH_4Cl , 50 mg/L kanamycin and 150 $\mu\text{L/L}$ of a solution containing 40.00 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10.00 g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 10.00 g/L $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, 7.30 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 2.00 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.00 g/L $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 1.00 g/L $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.50 g/L H_3BO_3 .

For bioreactor cultivations, defined minimal media according to DeLisa et al. [42] 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). CO_2 and O_2 in the off-gas were analyzed with an off-gas analyzer (M. Müller AG, 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. 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. Off-gas composition was analyzed with DASGIP GA gas analyzer (Eppendorf).

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 substrate uptake on physiology, productivity, lysis and leakiness during SpA production, a full-factorial screening Design of Experiments (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 all cultures were far exceeded (manifested in glucose accumulation and lysis) 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 72 h. 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% H₃PO₄ and the flow rate constant at 0.5 mL/min.

The biomass yield on glucose 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.

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

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

Analysis Of Lysis

Under the assumption that released DNA is proportional to amount of lysed cells, quantification of lysis was adapted from Klein et al. [43]. For our calculations, we assumed a cellular DNA content of 31 mg/g, which was taken from literature [44]. 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, which was determined by incubating the cell free culture supernatant at 30 °C and measuring the DNA concentration every 30–60 min. This resulted in a constant

degradation rate of $9.2 \pm 1.5\%/h$, with respect to initial DNA concentration (see Additional File 3). The amount of lysed cells in percent were calculated as shown in Equations 3–5:

$$r_{X,l,n} = \left(\frac{c_{DNA,n} - c_{DNA,n-1}}{t_n - t_{n-1}} + r_{d,DNA} * \Delta t \right) * \frac{1}{w_{X,DNA}} \quad (3)$$

$$x_{l,n} = x_{l,n-1} + \frac{(r_{X,l,n} + r_{X,l,n-1}) * (t_n - t_{n-1})}{2} \quad (4)$$

$$lysed\ cells = \frac{x_l}{x_l + x} * 100 \quad (5)$$

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, 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 minutes (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) was diluted in 2 × Laemmli buffer. The samples and standard were then incubated at 95 °C 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 Eq. 5:

$$leakiness = \frac{w_{Pin}}{w_{Pex} + w_{Pin}} * 100 \quad (7)$$

Abbreviations

$q_{S,0}$

biomass specific glucose uptake rate at beginning of induction in g/g/h

SpA

IgG binding domain of *Staphylococcus aureus* Protein A

T

Cultivation temperature in °C

VHH

VHH single domain antibody

w_{Pex}

biomass specific extracellular titer of recombinant product in mg/g

w_{Pin}

biomass specific intracellular titer of recombinant product in mg/g

$Y_{X/S}$

biomass yield on glucose

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

Competing interests

The authors declare no conflict of interests.

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Authors' contributions

JK contributed to the design of the experiments, performed acquisition, analysis and interpretation of data and prepared the manuscript. LR contributed to acquisition, analysis and interpretation of data. LF contributed to acquisition of data. JM and OS contributed to the design of the experiments and revision of the manuscript.

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Figures

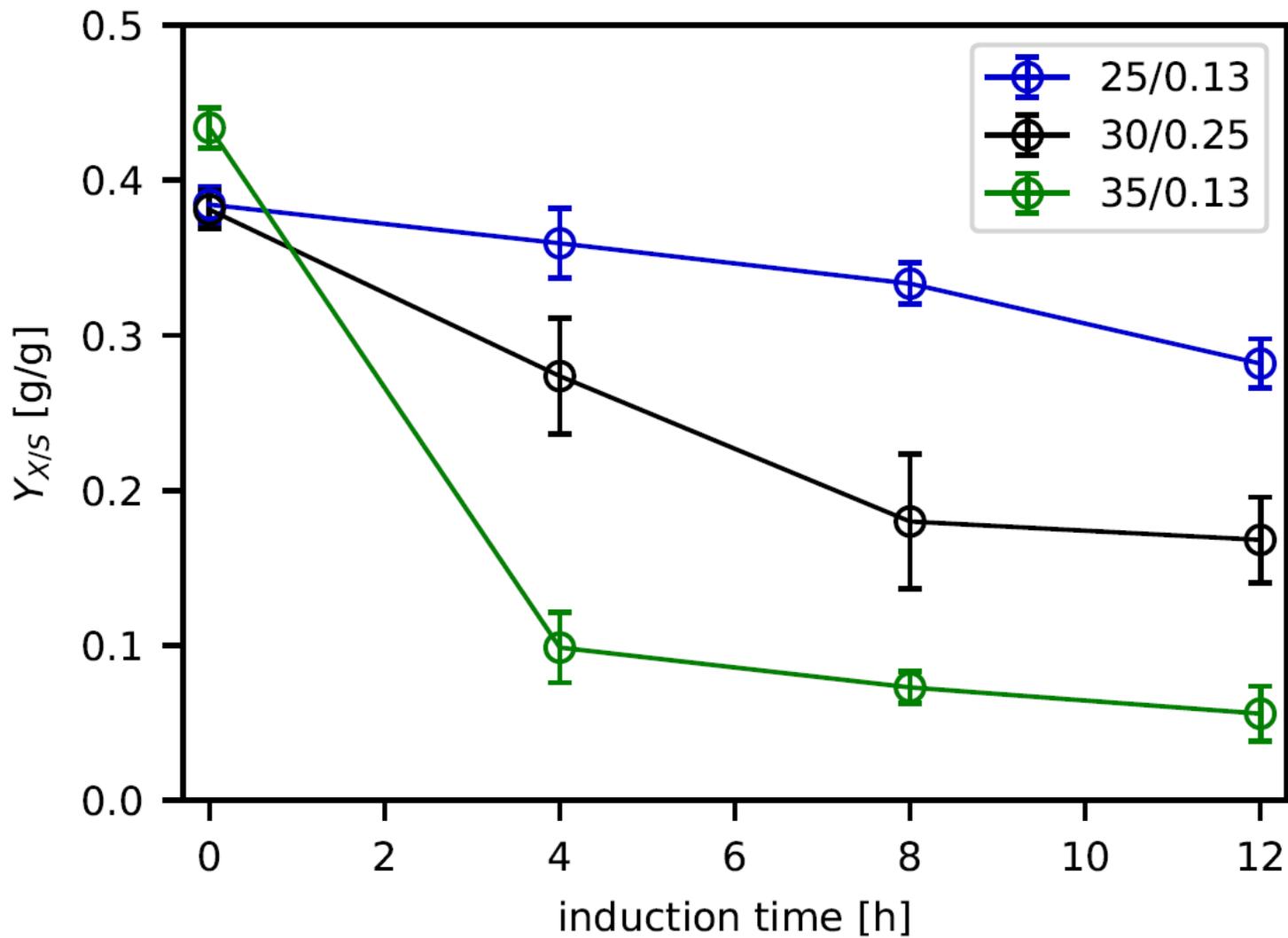


Figure 1

Biomass yield in cultivations of BL21(DE3) producing SpA.

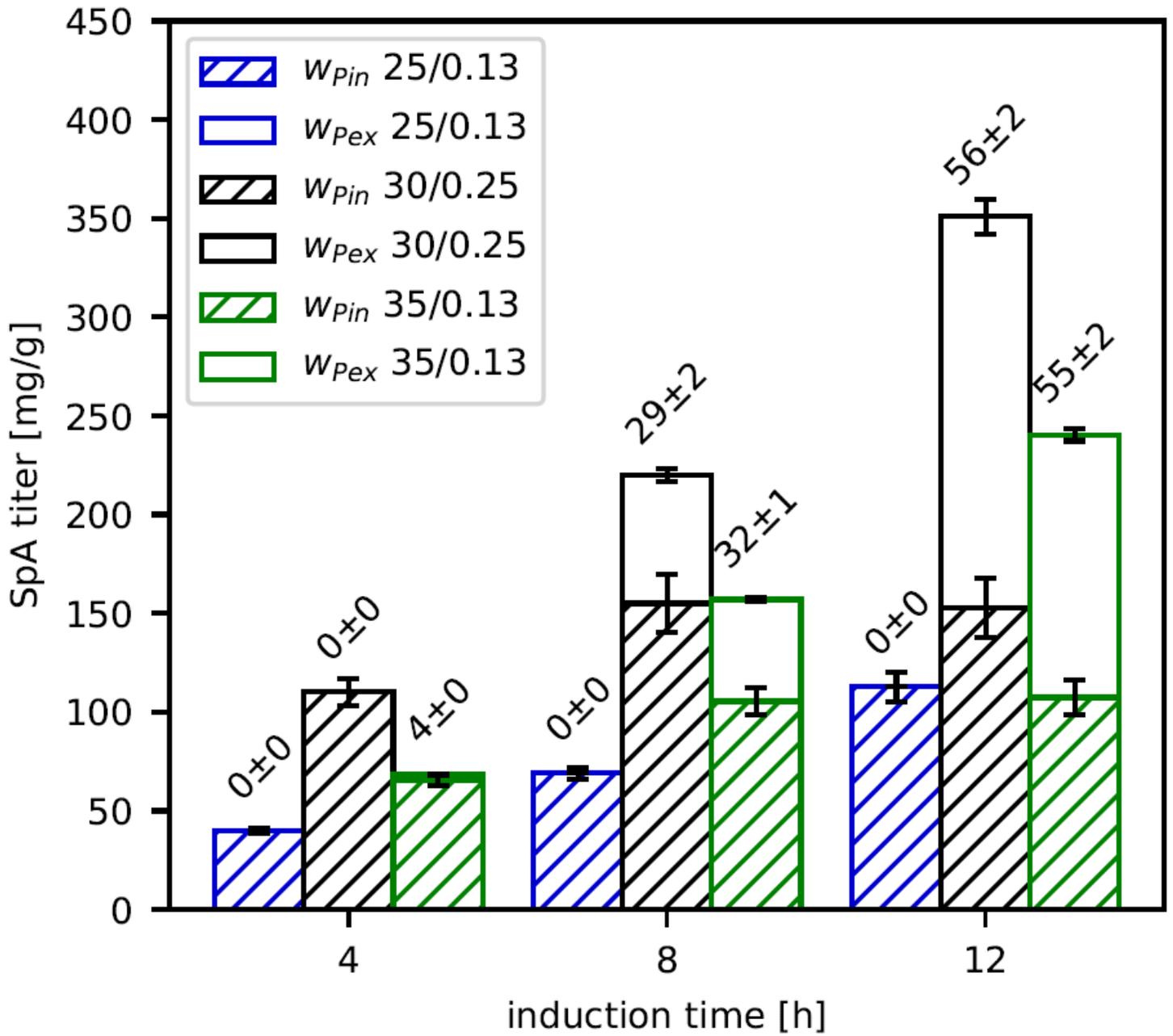


Figure 2

Intra- and extracellular soluble SpA titer in cultivations of BL21(DE3). Annotations above the columns represent leakiness in percent.

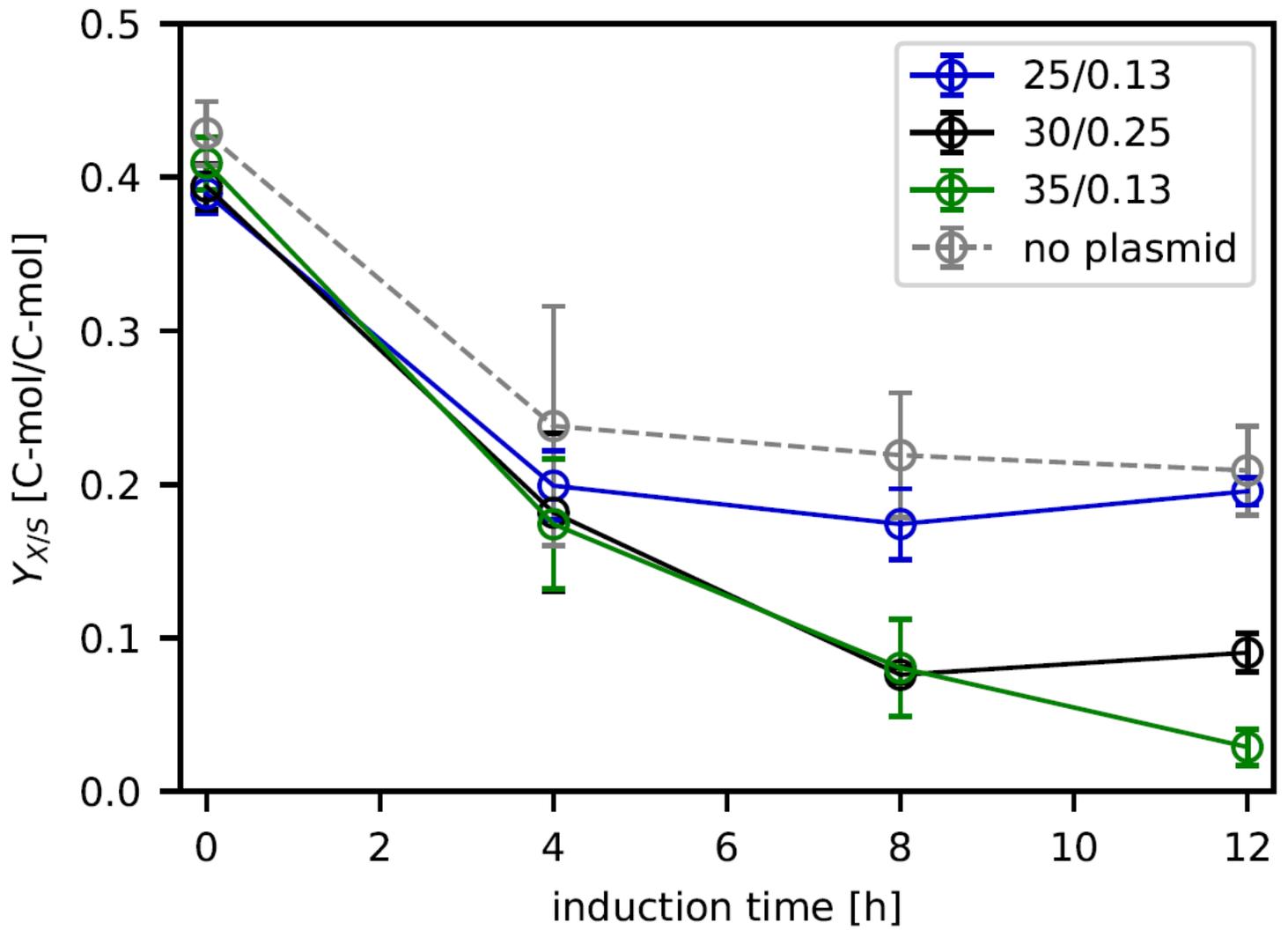


Figure 3

Biomass yield in cultivations of the X-press strain without plasmid and producing SpA.

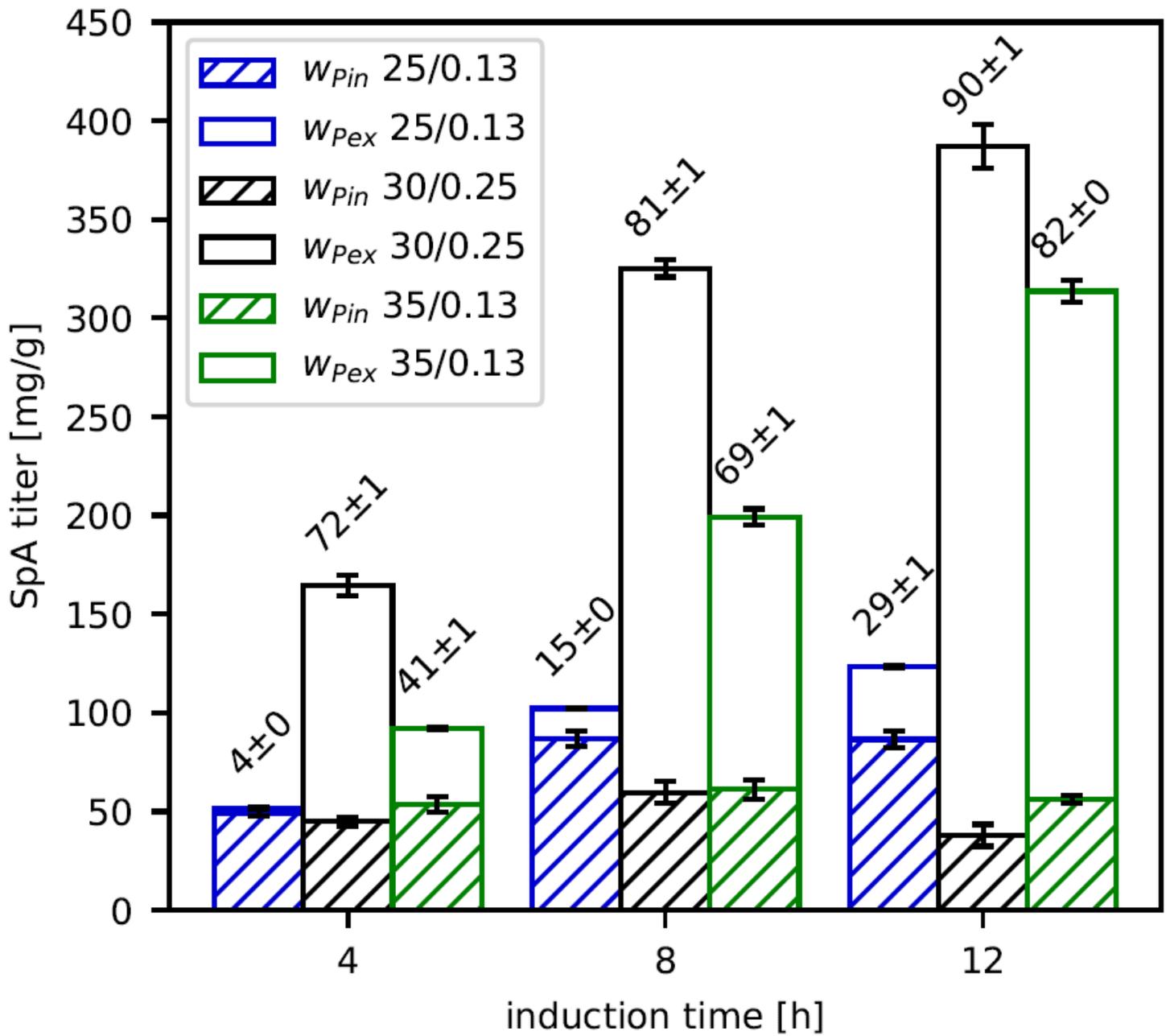


Figure 4

Intra- and extracellular soluble SpA titer in cultivations of the X-press strain. Annotations above the columns represent leakiness in percent.

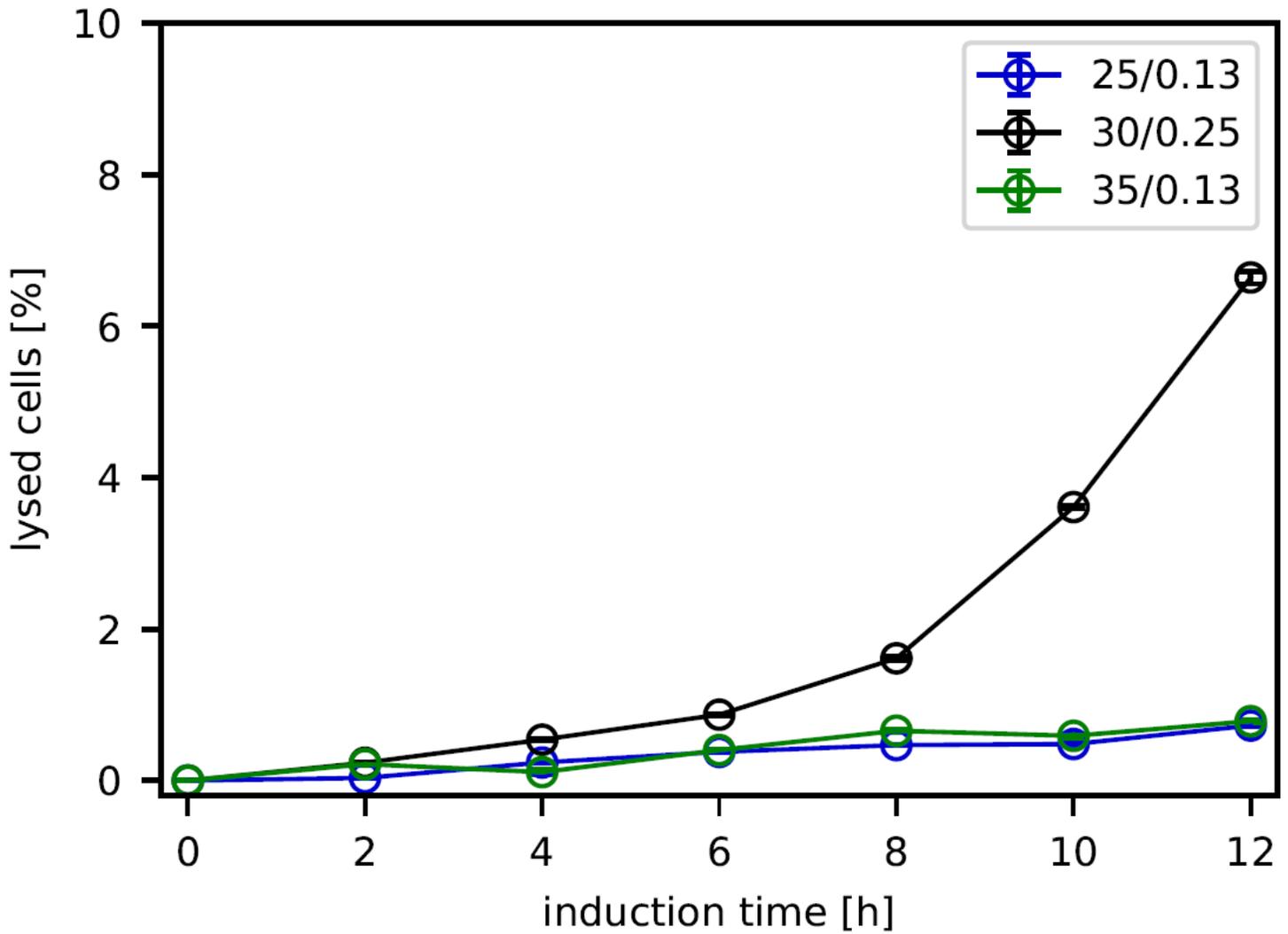


Figure 5

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

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