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DIPLOMARBEIT

Investigation of induction conditions for recombinant L-Threonine-Aldolase expression in *E. coli*

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unter der Anleitung von

Assoc. Prof. Dipl.-Ing. Dr.nat.techn Oliver Spadiut

und

Dipl.-Ing. Dr.techn. Johanna Hausjell

durch

Maximilian Clemens Winkler [redacted]

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Maximilian Clemens Winkler

Abstract

Many approaches have been investigated regarding recombinant protein production in *E. coli*, the most prominent being a pET plasmid in the strain *E. coli* BL21(DE3) with the target protein under control of the T7 promoter. However, other strains may be required for the expression of a target protein. The Austrian company Valanx Biotech GmbH approached the IBD group of TU Wien to find a production process for recombinant L-Threonine Aldolase (rLTA) in *E. coli* BL21 with the target gene under control of the phage-derived T5 promoter. This process would then be implemented in a scale-up experiment. Thus, different conditions for the induction of rLTA expression were investigated in this particular expression system. It was hypothesized that active soluble rLTA expression and the productivity of the expression system for this protein is dependent on (1) the type of inducer, (2) the temperature during induction and (3) the substrate feeding rate translating into a specific growth rate during induction. It was shown that the inducer lactose with a specific growth rate of $\mu = 0.05\ h^{-1}$ and an induction temperature of 30 °C yielded the most promising results with respect to active soluble rLTA production.

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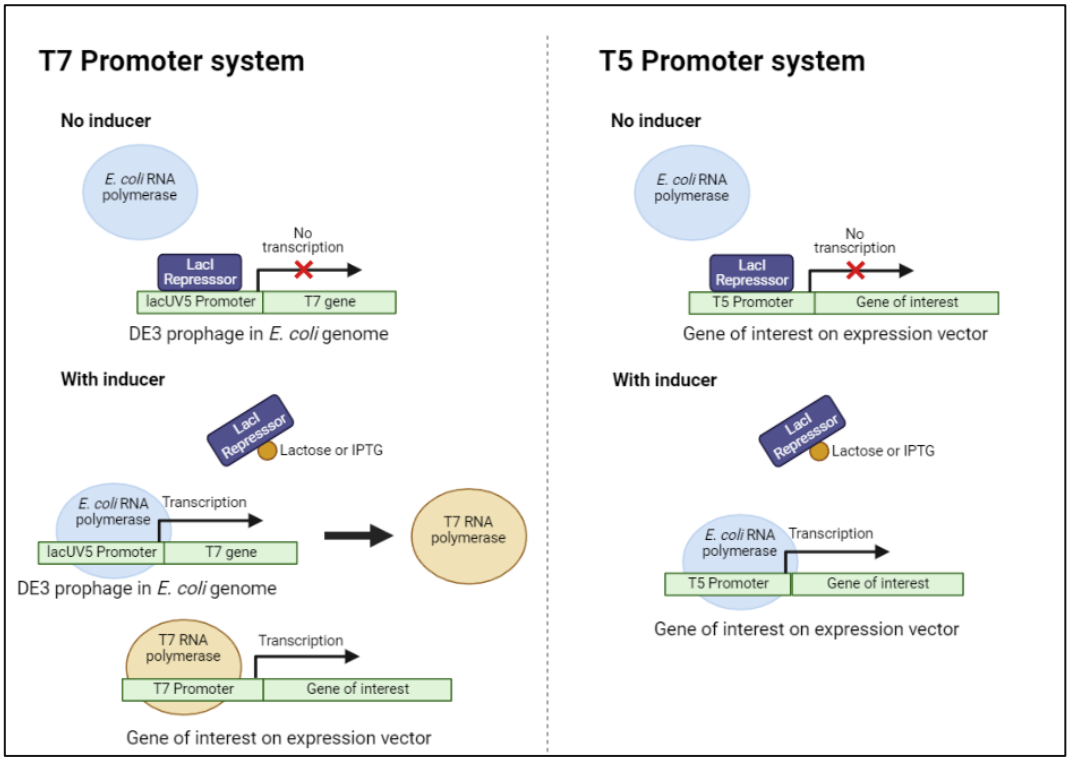
Introduction

One of the most widely used recombinant protein expression systems is the *E. coli* strain BL21(DE3) [1,2] harboring a pET plasmid [3–5]. This expression system uses a T7 promoter controlling target protein expression in an *E. coli* strain with the DE3 prophage on the chromosome, containing the T7 RNA polymerase gene. This system was first developed by Studier et al. in the eighties. [6] Since then, it has become a benchmark in *E. coli* recombinant protein production systems. Due to the absence of the T7 RNA polymerase gene in the chromosome of wild type *E. coli*, the T7 promoter can only be used to control target protein expression in strains which harbor the gene that encodes this RNA polymerase on the chromosome. The native RNA polymerase of *E. coli* BL21 does not recognize the T7 promoter as such. The expression of RNA polymerase encoded in the DE3 prophage in the *E. coli* BL21(DE3) genome is induced by lactose or similar compounds, since the T7 RNA polymerase gene is under control of a lacUV5 promoter. [7] This system gives the possibility to tightly control target protein expression by also constitutively expressing T7 lysozyme on a separate plasmid [8] which targets basal T7 RNA polymerase activity without inducer present.

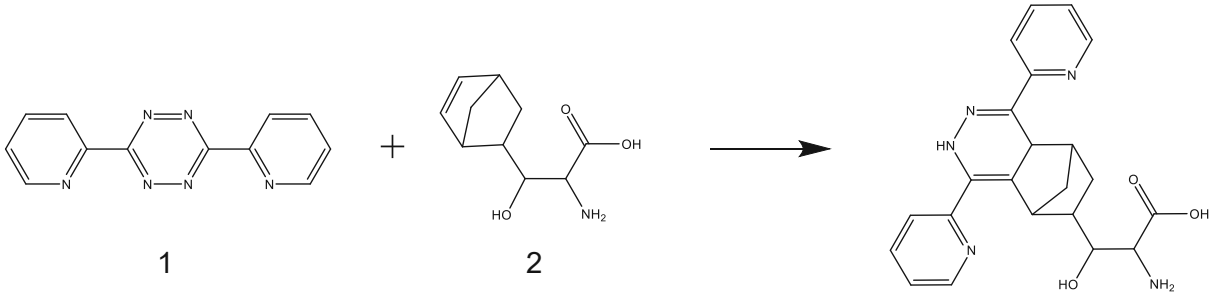
E. coli BL21(DE3) harboring a pET plasmid with T7 promoter control over target protein expression has become an established host for recombinant pharmaceutical production in the large scale. [9] A lac-derived promoter controlling recombinant target protein expression might be a viable alternative without the need of this specific strain. One of these alternatives is represented by the T5 promoter. [10] This phage-derived promoter is recognized by *E. coli*'s native RNA polymerase. [11] Similar to the T7 expression system, target gene expression under control of the T5 promoter can be induced by addition of lactose or Isopropyl β-d-1-thiogalactopyranoside (IPTG) to the cultivation media. Target gene expression under control of this promoter is possible in strains without the DE3 prophage on the chromosome, giving way to opportunities of recombinant protein production in strains with other desirable genotypes than that of BL21(DE3). A comparative overview of the T7 and the T5 promoter is shown in Figure 1.

Figure 1: T7 vs. T5 promoter system. The T7 promoter requires the T7 RNA polymerase encoded in the DE3 prophage for which the expression of the gene is induced by lactose or IPTG through binding the Lac repressor. The T5 promoter is recognized by E. coli's native RNA polymerase which can transcribe the gene of interest after induction with lactose or IPTG. The figure was made with BioRender.



L-Threonine Aldolase from *Pseudomonas putida* (LTA-1, UniProtKB O50584) [12–14] is an enzyme that can catalyze the cleavage reaction of DL-Phenylserine to Benzaldehyde and Glycine. LTA-1 shows promiscuous enzyme activity, which enables it to catalyze formation or cleavage reactions of similar substrates. One particular compound of interest that can be formed by this enzyme is the non-canonical amino acid (ncAA) L-3-Norbornylserine (NorI) [15], which was developed at Graz University of Technology with subsequent IP transfer to Valanx Biotech GmbH. This ncAA is attractive due to its side chain which is able to undergo very fast and specific so-called copper-free inverse-electron demand Diels-Alder (iEDDA) "click" reactions with a suitable reaction partner, such as 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine (Scheme 1). This reaction chemistry can be used as linkers for functionalization of biopolymers [16] as well as bioconjugation. [17]

Scheme 1: Copper-free inverse-electron demand Diels-Alder (iEDDA) "click" chemistry reaction between 3,6-di(pyridin-2-yl)-1,2,4,5-tetrazine (1) and NorI (2)



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Goal & Hypotheses

The goal of this work was to find cultivation conditions yielding high productivity for active and soluble LTA-1 production in *E. coli* since this enzyme is of particular interest for the synthesis of NorI. Of the investigated conditions, one condition was to be chosen to implement a scale-up experiment. The *E. coli* strain BL21 with the target gene under control of the T5 promoter was the expression system of choice (Figure 10), which does not require a chromosomal copy of the T7 RNA polymerase. The fermentation experiment strategy was set within the framework of a controlled bioprocess environment in bioreactors using defined media for reasons of process control and reproducibility.

In light of these constraints, following hypotheses were formulated:

Active soluble LTA-1 expression and productivity of the expression system for this enzyme is dependent on

(1) the type of inducer (IPTG and Lactose, respectively).

The T5 promoter is inducible by both Lactose and IPTG. In the T5 promoter sequence, three lac operators (*lacO*) interact with the lac repressor protein LacI, inhibiting the RNA polymerase from transcribing the target gene. By adding Lactose or IPTG, LacI binds preferentially to the inducer and releases the *lacO* sequences in the promoter, allowing the RNA polymerase to initiate target gene transcription. To reduce basal expression of LTA-1 without any inducer present, the $lacI^q$ promoter is used for *lacI* expression. This promoter displays a point mutation compared to the wild type, increasing LacI expression by 10-fold. [18] IPTG is pulsed once at the beginning of the induced fed-batch phase, whereas Lactose is fed to the reactor in a mixed-feed regime throughout the induction phase. The effect of inducer type and addition method was investigated by performing experiments with those respective inducers in order to test the first hypothesis of this thesis.

(2) the temperature during the induced fed-batch.

Cultivation temperature can have a major effect on recombinant protein production in *E. coli.* [19–21] Not only does cultivation temperature influence the growth behavior and morphology of *E. coli* [22], it has been shown to influence the formation of inclusion bodies (IBs). [23,24] In order to test the hypothesis whether the temperature during the induced fed-batch phase of the bioprocess influences the expression of active soluble LTA-1, cultivation experiments with 30 °C and 37 °C induction temperature were performed.

(3) the substrate feeding rate during the induced fed-batch.

In order to not overfeed *E. coli* with substrate while producing the target protein, a substrate-limited fed-batch strategy is often times employed, where the substrate feeding regime controls the specific growth rate directly, following Monod's model of growth kinetics. [25] In this case, when looking at the two different induces chosen for investigation, a feed with only glucose as carbon source along with singly-pulsed IPTG induction is compared to a mixed feed regime comprised of glucose and lactose, where lactose acts as inducer [4,26]. The substrate feeding rate has been shown to be a factor influencing target protein production. [27–29] The hypothesis that the substrate feeding rate also influences active and soluble LTA-1 production will be investigated in this thesis.

References

[1] G.L. Rosano, E.A. Ceccarelli, Recombinant protein expression in Escherichia coli: Advances and challenges, Front. Microbiol. 5 (2014). https://doi.org/10.3389/fmicb.2014.00172.

[2] G.L. Rosano, E.S. Morales, E.A. Ceccarelli, New tools for recombinant protein production in *Escherichia coli* : A 5-year update, Protein Sci. 28 (2019) 1412–1422. https://doi.org/10.1002/pro.3668.

[3] A.H. Rosenberg, B.N. Lade, C. Dao-shan, S.W. Lin, J.J. Dunn, F.W. Studier, Vectors for selective expression of cloned DNAs by T7 RNA polymerase, Gene. 56 (1987) 125–135. https://doi.org/10.1016/0378-1119(87)90165-X.

[4] D.J. Wurm, J. Quehenberger, J. Mildner, B. Eggenreich, C. Slouka, A. Schwaighofer, K. Wieland, B. Lendl, V. Rajamanickam, C. Herwig, O. Spadiut, Teaching an old pET new tricks: tuning of inclusion body formation and properties by a mixed feed system in E. coli, Appl. Microbiol. Biotechnol. 102 (2018) 667–676. https://doi.org/10.1007/s00253-017-8641-6.

[5] P.J. Shilling, K. Mirzadeh, A.J. Cumming, M. Widesheim, Z. Köck, D.O. Daley, Improved designs for pET expression plasmids increase protein production yield in Escherichia coli, Commun. Biol. 3 (2020) 1–8. https://doi.org/10.1038/s42003-020-0939-8.

[6] F.W. Studier, B.A. Moffatt, Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes, J. Mol. Biol. 189 (1986) 113–130. https://doi.org/10.1016/0022-2836(86)90385-2.

[7] J.W. Dubendorf, F.W. Studier, Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with lac repressor, J. Mol. Biol. 219 (1991) 45–59. https://doi.org/10.1016/0022-2836(91)90856-2.

[8] F.W. Studier, Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system, J. Mol. Biol. 219 (1991) 37–44. https://doi.org/10.1016/0022-2836(91)90855-Z.

[9] C.J. Huang, H. Lin, X. Yang, Industrial production of recombinant therapeutics in Escherichia coli and its recent advancements, J. Ind. Microbiol. Biotechnol. 39 (2012) 383–399. https://doi.org/10.1007/s10295-011-1082-9.

[10] R. Gentz, H. Bujard, Promoters recognized by Escherichia coli RNA polymerase selected by function: Highly efficient promoters from bacteriophage T5, J. Bacteriol.

164 (1985) 70–77. https://doi.org/10.1128/jb.164.1.70-77.1985.

[11] A. Schuller, M. Cserjan-Puschmann, C. Tauer, J. Jarmer, M. Wagenknecht, D. Reinisch, R. Grabherr, G. Striedner, Escherichia coli σ 70 promoters allow expression rate control at the cellular level in genome-integrated expression systems, (n.d.). https://doi.org/10.1186/s12934-020-01311-6.

[12] J. Steinreiber, K. Fesko, C. Reisinger, M. Schürmann, F. van Assema, M. Wolberg, D. Mink, H. Griengl, Threonine aldolases-an emerging tool for organic synthesis, Tetrahedron. 63 (2007) 918–926. https://doi.org/10.1016/j.tet.2006.11.035.

[13] K. Fesko, M. Uhl, J. Steinreiber, K. Gruber, H. Griengl, Biocatalytic access to a,a-dialkyl-a-amino acids by a mechanism-based approach, Angew. Chemie - Int. Ed. 49 (2010) 121–124. https://doi.org/10.1002/anie.200904395.

[14] N. Dückers, K. Baer, S. Simon, H. Gröger, W. Hummel, Threonine aldolases-screening, properties and applications in the synthesis of non-proteinogenic β-hydroxy-α-amino acids, Appl. Microbiol. Biotechnol. 88 (2010) 409–424. https://doi.org/10.1007/s00253-010-2751-8.

[15] M. Lukesch, WO2019016354A1 - Novel amino acids bearing a norbornene moiety - Google Patents, (n.d.). https://patents.google.com/patent/WO2019016354A1/en?oq=U.S.+Patent+WO2019016354A1 (accessed December 31, 2021).

[16] C. Wappl, V. Schallert, C. Slugovc, A.C. Knall, S. Spirk, Highly norbornylated cellulose and its “Click” modification by an inverse-electron demand diels-alder (iEDDA) reaction, Molecules. 26 (2021). https://doi.org/10.3390/molecules26051358.

[17] A.C. Knall, C. Slugovc, Inverse electron demand Diels-Alder (iEDDA)-initiated conjugation: A (high) potential click chemistry scheme, Chem. Soc. Rev. 42 (2013) 5131–5142. https://doi.org/10.1039/c3cs60049a.

[18] M.P. Calos, DNA sequence for a low-level promoter of the lac repressor gene and an “up” promoter mutation, Nature. 274 (1978) 762–765. https://doi.org/10.1038/274762a0.

[19] M. Mühlmann, E. Forsten, S. Noack, J. Büchs, Optimizing recombinant protein expression via automated induction profiling in microtiter plates at different temperatures, Microb. Cell Fact. 16 (2017) 220. https://doi.org/10.1186/s12934-017-0832-4.

[20] M. Gadgil, V. Kapur, W.S. Hu, Transcriptional response of Escherichia coli to

temperature shift, Biotechnol. Prog. 21 (2005) 689–699. https://doi.org/10.1021/bp049630l.

[21] C. Slouka, J. Kopp, S. Hutwimmer, M. Strahammer, D. Strohmer, E. Eitenberger, A. Schwaighofer, C. Herwig, Custom made inclusion bodies: Impact of classical process parameters and physiological parameters on inclusion body quality attributes, Microb. Cell Fact. 17 (2018) 1–15. https://doi.org/10.1186/s12934-018-0997-5.

[22] P. Kumar, A. Libchaber, Pressure and temperature dependence of growth and morphology of escherichia coli: Experiments and stochastic model, Biophys. J. 105 (2013) 783–793. https://doi.org/10.1016/j.bpj.2013.06.029.

[23] L. Strandberg, S.O. Enfors, Factors influencing inclusion body formation in the production of a fused protein in Escherichia coli, Appl. Environ. Microbiol. 57 (1991) 1669–1674. https://doi.org/10.1128/aem.57.6.1669-1674.1991.

[24] J. Kopp, A.-M. Kolkmann, P.G. Veleenturf, O. Spadiut, C. Herwig, C. Slouka, Boosting Recombinant Inclusion Body Production—From Classical Fed-Batch Approach to Continuous Cultivation, Front. Bioeng. Biotechnol. 7 (2019) 297. https://doi.org/10.3389/fbioe.2019.00297.

[25] H. Chmiel, ed., Bioprozesstechnik, Spektrum Akademischer Verlag, Heidelberg, 2011. https://doi.org/10.1007/978-3-8274-2477-8.

[26] D.J. Wurm, J. Hausjell, S. Ulonska, C. Herwig, O. Spadiut, Mechanistic platform knowledge of concomitant sugar uptake in Escherichia coli BL21(DE3) strains., Sci. Rep. 7 (2017) 45072. https://doi.org/10.1038/srep45072.

[27] M.G. Aucoin, V. McMurray-Beaulieu, F. Poulin, E.B. Boivin, J. Chen, F.M. Ardelean, M. Cloutier, Y.J. Choi, C.B. Miguez, M. Jolicoeur, Identifying conditions for inducible protein production in E. coli: Combining a fed-batch and multiple induction approach, Microb. Cell Fact. 5 (2006) 27. https://doi.org/10.1186/1475-2859-5-27.

[28] K. Ukkonen, S. Mayer, A. Vasala, P. Neubauer, Use of slow glucose feeding as supporting carbon source in lactose autoinduction medium improves the robustness of protein expression at different aeration conditions, Protein Expr. Purif. 91 (2013) 147–154. https://doi.org/10.1016/j.pep.2013.07.016.

[29] P. Wechselberger, P. Sagmeister, H. Engelking, T. Schmidt, J. Wenger, C. Herwig, Efficient feeding profile optimization for recombinant protein production using physiological information, Bioprocess Biosyst. Eng. 35 (2012) 1637–1649. https://doi.org/10.1007/s00449-012-0754-9.

Paper draft: Investigation of induction conditions for fed-batch cultivations using a T5 promoter-based *E. coli* BL21 expression system

Maximilian Winkler[1,2], Johanna Hausjell[1], Regina Kutscha[1], Oliver Spadiut[1*]

[1]*Institute of Chemical Engineering, Vienna University of Technology*

[2]*Valanx Biotech GmbH*

[*]*Correspondence* oliver.spadiut@tuwien.ac.at

Abstract

Established expression systems, such as *E. coli* BL21(DE3) often use the T7 promoter on a pET plasmid for target gene expression. The phage-derived, lactose- and IPTG-inducible T5 promoter can be directly used in *E. coli* strains not harboring a chromosomal copy of the T7 RNA polymerase gene, since the T5 promoter is recognized by *E. coli*'s native RNA polymerase. In order to reduce basal expression of target protein, LacI must be abundant in the cell, which can be achieved by using the *lacI*^{q} promoter for *lacI* expression. An expression system comprising of *E. coli* BL21 harboring a pQE plasmid with the target gene under control of the T5 promoter, as well as enhanced Lac repressor expression by *lacI*^{q}, was investigated. It was hypothesized that the productivity of the expression system with respect to the target protein, recombinant L-Threonine Aldolase, is dependent on the type of inducer, substrate feed rate during induction and induction temperature. It was demonstrated that under some conditions, productivity remains stable after 8 h of induction. Thus, this expression system is a suitable alternative to the T7 promoter in *E. coli* BL21(DE3) for high cell density fermentations.

Keywords: recombinant protein expression, Escherichia coli, T5 promoter, lac repressor, fed-batch

Introduction

A variety of vectors have been used for recombinant protein expression in *E. coli*. In particular, the pET system based on the T7 promoter [7,8,26,30–33] and the pQE system based on the T5 promoter have been used excessively. [11,31,34–36] The T7 promoter is recognized by the lambda T7 RNA polymerase, whereas the T5 promoter is recognized by *E. coli*'s native RNA polymerase. The T5 promoter enables recombinant protein expression without the need of a gene encoding the T7 RNA polymerase, competing strongly for the *E. coli* RNA polymerase with other promoters. [10] Thus, the T5 promoter system is compatible with *E. coli* strains that do not have the DE3 prophage incorporated in the genome.

It is necessary to repress the transcription of the T5-controlled target gene tightly, as not to overburden the cell metabolically before induction of target gene expression. An appropriate amount of lac repressor protein LacI must be present in order to achieve sufficient repression of the T5 promoter to ensure controllable induction. When no or insufficient repressor is present, the cells are burdened by basal expression of target protein before induction [35]. One approach to increase the amount of LacI in the cell is to use the $lacI^q$ promoter for *lacI* gene expression. This promoter has one point mutation compared to the wild type, increasing expression 10-fold. [18] Blommel et al. used a combination of the T5 promoter with two adjacent lac operators controlling the target gene expression and the $lacI^q$ promoter controlling *lacI* expression to investigate the influence of LacI abundance on recombinant protein expression and carbon source uptake. They showed that an increase of LacI shifts the carbon source uptake in autoinduction medium to preferably consume glycerol over lactose after glucose depletion. [34] Kawe et al. also investigated the combination of T5 promoter and $lacI^q$ promoter to show that when Lac repressor is not overexpressed, T5 promoter deletion occurs. [35] The $lacI^q$ promoter for the lac repressor can also be used to downregulate basal expression of proteins toxic to *E. coli*. [1,37] Due to a high abundance of lac repressor, lac-derived promoter regions are blocked more effectively before induction. [38] Duebendorf et al. showed that one chromosomal copy is not enough to repress all operators on a multicopy plasmid. [7] To completely curb basal expression of a gene of interest under a T5 promoter, up to three lac operators have to be used together with $lacI^q$, as shown by Schuller. [11]

Traditional induced fed-batch strategies at glucose-limiting conditions may lead to protein degradation and mRNA instability. [39] T5-promoter systems in *E. coli* have been shown to suffer from drops in specific productivity after only short induction times when induced with IPTG. It is suspected that the use of a strong promoter such as T5 may lead to a high metabolic burden and inhibition of cell growth. [36]

In order to explore the T5 promoter as a viable alternative to established promoters such as T7, we constructed an expression system with *E. coli* BL21 using a plasmid with a recombinant target gene under control of the T5 promoter and *lacI* under $lacI^q$ promoter control. We wanted to shed light on this particular expression system by investigating the influence of (I) the type of inducer, (II) the specific growth rate and (III) the temperature during induction. Among the investigated induction conditions, one was investigated further in a scale-up experiment. As a model protein recombinant L-threonine aldolase (rLTA) from *Pseudomonas putida* was used. Its quaternary structure is homotetrameric, with each monomer being 37.98 kDa in size. [40] This protein is able to catalyze the cleavage reaction from L-erythro-phenylserine and L-threo-phenylserine to glycine and benzaldehyde, as well as from L-allo-threonine and L-threonine to glycine and acetaldehyde. [41]

Materials & methods

E. coli strain and plasmid

The gene coding for L-TA was cloned into a pQE-80L plasmid backbone (Qiagen, Hilden, Germany). The pQE backbone contains a ColE1 origin, a β-lactamase gene as well as a lac repressor gene with a $lacI^q$ promoter. L-TA expression is induced by a T5-promoter with two adjacent lac operators. [42] The plasmid map of pQE-LTA1 is shown in Figure 10. The *E. coli* BL21 strain of genotype F-, ompT, hsdS (rB -, mB -), gal, dcm (GE Healthcare) was made chemically competent and transformed [43] with the plasmid pQE-LTA1.

Small scale bioreactor cultivations

For each inducer, IPTG and lactose respectively, four cultivations were carried out in parallel. The cultivation system used was a DASbox® Mini Bioreactor (Eppendorf, Hamburg, Germany) system. The off-gas was analyzed for CO_2 and O_2 with a DASGIP® GA gas analyzer. Dissolved oxygen (DO) saturation was measured with a Visiferm DO 120 electrode (Hamilton, Reno, NV, USA). During the fermentation, DO was kept above 40 %, which was achieved by a supply of 2 vvm oxygen-air-mixture. pH control of the fermentation broth was done by supplying 12.5 % NH_4OH. DASware® software was used to monitor and control the process parameters.

For the preculture, 500 mL DeLisa overnight culture medium [44] containing 8 g/L glucose, pH 7.2, supplied with 1 mg/L Ampicillin, was inoculated with 1 mL from cell stocks frozen at – 80 °C and cultivated over night for 16 h at 37 °C in an Infors HR Multitron Shaker (Infors, Bottmingen, Switzerland). The main culture was carried out in bioreactors containing 150 mL of DeLisa batch medium [44] supplied with 10 g/L glucose and 1 mg/L Ampicillin. Each was inoculated with 15 mL of preculture. After a batch phase, where all glucose in the medium was consumed, a fed-batch with DeLisa feed medium [44] containing 400 g/L glucose was initiated and run until a biomass concentration of 25 g/L dry cell weight was reached. Then, an induced

fed-batch was initiated for a duration of 12 h. For the induced Fed Batch, pH was set to 7. The induction temperature and specific growth rate setpoints for each run are shown in Table 1. Induction with IPTG was performed by pulsing to a concentration of 100 mM and DeLisa feed medium [44] containing 400 g/L glucose was fed. For lactose induction, first an adaption pulse to 20 g/L lactose performed, then, immediately after pulsing, DeLisa feed medium [44] containing 150 g/L lactose and 200 g/L glucose was fed. Experimental data showed that *E. coli* BL21(DE3) requires 4 h of lactose excess until reaching the maximum lactose specific uptake rate while still feeding glucose [26,33]. This strategy was adapted for the cultivations with lactose induction. Thus, a lactose pulse was performed as described above. The mixed feed containing glucose and lactose was used throughout the induced fed batch instead of switching from a mixed feed to a lactose-only feed after 4 h.

The feeding was regulated by a feed forward strategy: The set specific growth rate μ [h^{-1}] is directly proportional to the substrate uptake rate q_s over an estimated yield of 0.35 g_x/g_{Glc} based on experimental data from the research group (unpublished data), which in turn can be set by a substrate feed rate[45]. Calculation of the feed rate was performed with Equations 1 and 2.

$$F_0 = \frac{x_0 * V_0 * q_{s,Glc}}{w}$$

Equation 1: Initial feed rate calculation for the fed batch

$$F_t = F_0 * e^{q_{s,Glc} * Y_{X/S} * t}$$

Equation 2: Feed rate calculation during the fed batch

F_0	.....	Initial feed rate [g_{Feed}/h]
x_0	.....	Initial dry cell mass concentration [g_x/L]
V_0	.....	Initial reaction volume [L]
$q_{s,glc}$	.....	Specific glucose uptake rate [$g_{glc}/g_x/h$]
w	.....	Mass fraction of glucose in feed [g_{glc}/g_{feed}]
F_t	.....	Feed rate at time t after fed batch start [g_{feed}/h]
t	.....	Time after fed batch start [h]
$Y_{X/S}$	.....	Dry cell mass yield per substrate, 0.35 g_x/g_{Glc}

Table 1: Induction parameters for all DASbox fermentation runs with two different inducers IPTG and Lactose

Condition	T [°C]	μ [h^{-1}]
A	30	0.1
B	25	0.05
C	25	0.1
D	30	0.05

Samples for the analysis of dry cell weight (DCW), optical density at 600 nm (OD_{600}), sugar concentration, intra- and extracellular protein concentration and enzyme activity were taken at the beginning and the end of the batch and fed-batch phase, as well as every two hours for the whole induction time span.

Analytics

DCW was measured gravimetrically. Sugar concentrations were measured with high-pressure liquid chromatography. The used methods are described by Hausjell et al. [33]

For the measurement of enzyme activity, the sample cell pellets were resuspended to 1 mL and diluted with 1XPBS to an OD_{600} of 0.5. Then, this dilution was centrifuged, and the pellet lysed with 100 µL of BugBuster Mastermix (Merck, Darmstadt, Germany).

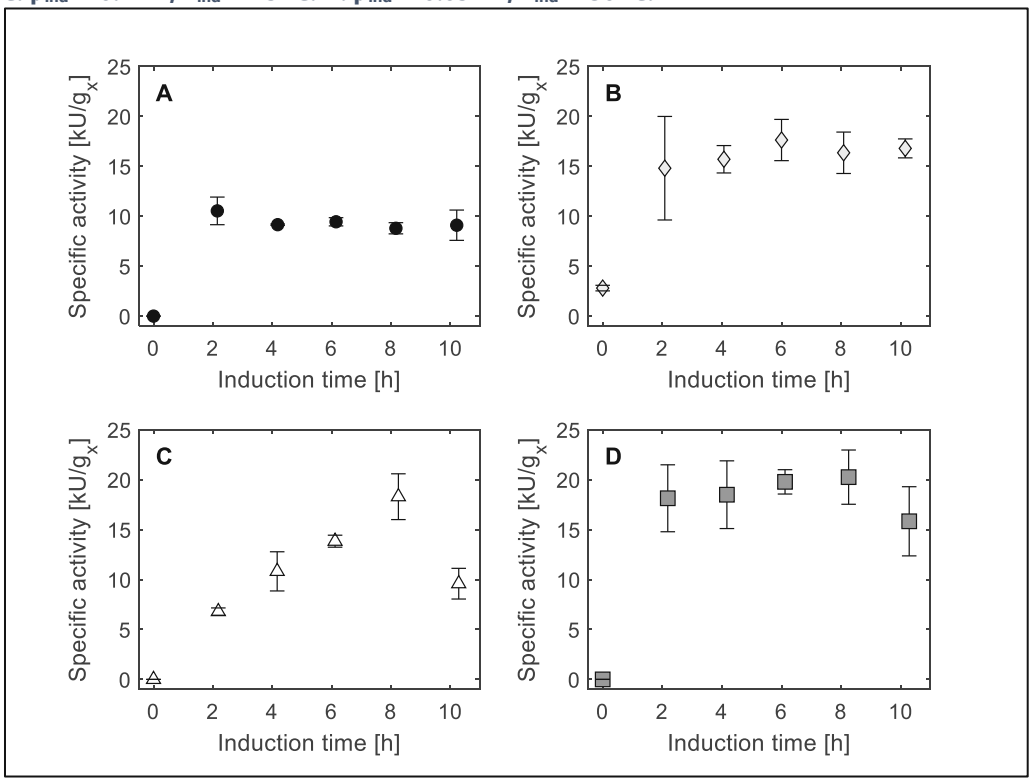
To measure enzymatic activity, an assay was established based on the works of Fesko et al.[46]. For this assay, which was performed with a Greiner BioOne UVStar 96 well plate (Greiner Bio-One, Kremsmünster, Austria) in a BioTek Synergy H1 plate reader (BioTek, Vermont, USA), 10 µL of lysate in the proper dilution in 1XPBS was mixed with 90 µL of 50 mM DL-Phenylserine and 2 µL of 2 mM Pyridoxal-5-phosphate subsequently and shaken vigorously for 10 s. Then, the initial turnover rate, which corresponds to the initial linear range of the absorption curve at 300 nm, was calculated. 300 nm is the absorption wavelength of Benzaldehyde, the cleavage product of DL-Phenylserine catalyzed by LTA.

Results and Discussion

As described above, the first approach to induction of recombinant LTA expression was implemented by pulsing IPTG to a final concentration of 1 mM in the reaction volume (Table 1).

In Figure 2, the specific intracellular enzyme activities and in Figure 3 the specific intracellular product formation rates of all fermentations with IPTG induction are shown.

Figure 2: Biomass-specific rLTA activity of IPTG induced cultures in the induction timespan.
A: μ_{ind} = 0.1 h^{-1}, T_{ind} = 30 °C. B: μ_{ind} = 0.05 h^{-1}, T_{ind} = 25 °C.
C: μ_{ind} = 0.1 h^{-1}, T_{ind} = 25 °C. D: μ_{ind} = 0.05 h^{-1}, T_{ind} = 30 °C.



The highest increase in specific activity occurs in the first two hours after induction. Since a 10-fold increase of LacI expression is expected due to the point mutation in the *lacI*^{q} promoter [47], IPTG may only be able to enter the cells by diffusion [48] at first, derepressing the lac operon by binding to LacI. This induces the expression of lactose permease expression from the *lacY* gene, initiating the transport of IPTG into the cells. Thereafter, active rLTA expression stays at a relatively constant level, except for condition C (high μ, low T), where an increase until 8h after induction can be seen, followed by a sharp decrease. Since energy metabolism is downregulated by lowering the cultivation temperature [20] from 37 °C in fed-batch phase to 25 °C in the induced fed batch phase, the applied substrate feeding rate might be too high, leading to glucose accumulation (Figure 5) in the bioreactor. This in turn may lead to carbon catabolite repression, as a high glucose concentration inhibits the formation of cyclic adenosine monophosphate (cAMP), which binds to the catabolite activator protein (CAP) to activate transcription by RNA polymerase at the promoter. [49] The biomass yield $Y_{x/s}$ [C-mol/C-mol] (Table 5) is subject to error for all IPTG-induced fermentations, thus a quantitative conclusion can hardly be drawn. However, the general rising trend of the biomass yield from 4 hours of induction towards the end of the induction period indicates that the metabolic burden [50] is decreasing for all conditions. This will be discussed towards the end of this chapter.

Figure 3: Productivity of IPTG induced cultures in kU per gram of biomass per hour during the induction timespan. The grey line indicates the zero line.
A: μ_{ind} = 0.1 h^{-1}, T_{ind} = 30 °C. B: μ_{ind} = 0.05 h^{-1}, T_{ind} = 25 °C.
C: μ_{ind} = 0.1 h^{-1}, T_{ind} = 25 °C. D: μ_{ind} = 0.05 h^{-1}, T_{ind} = 30 °C.

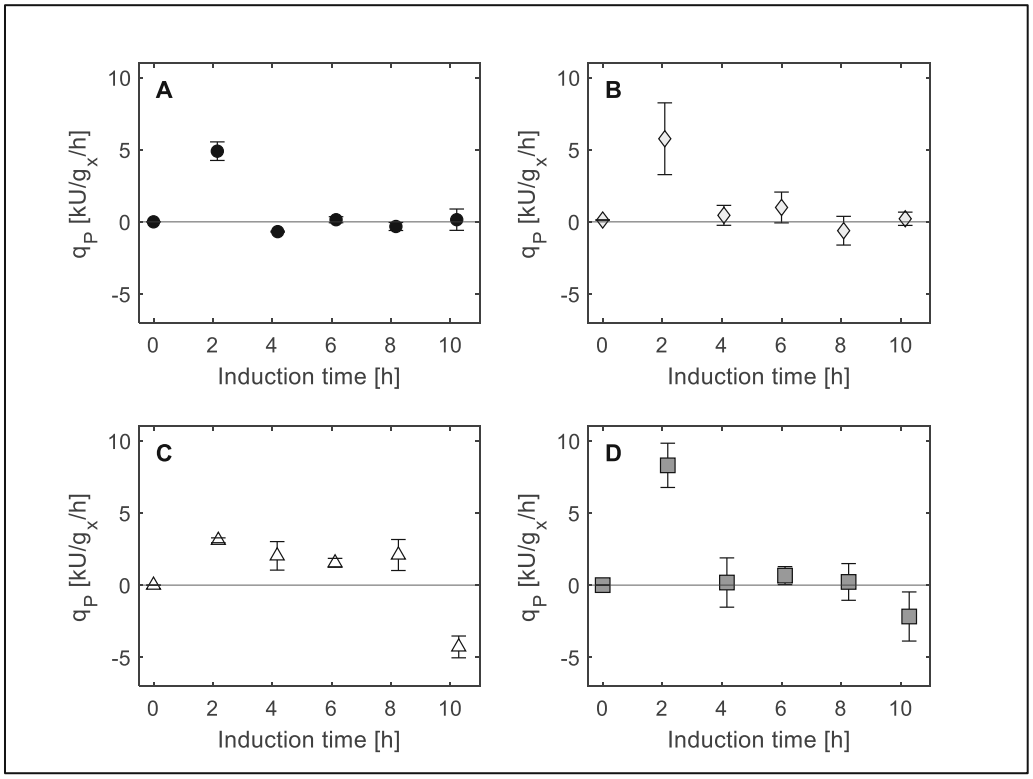
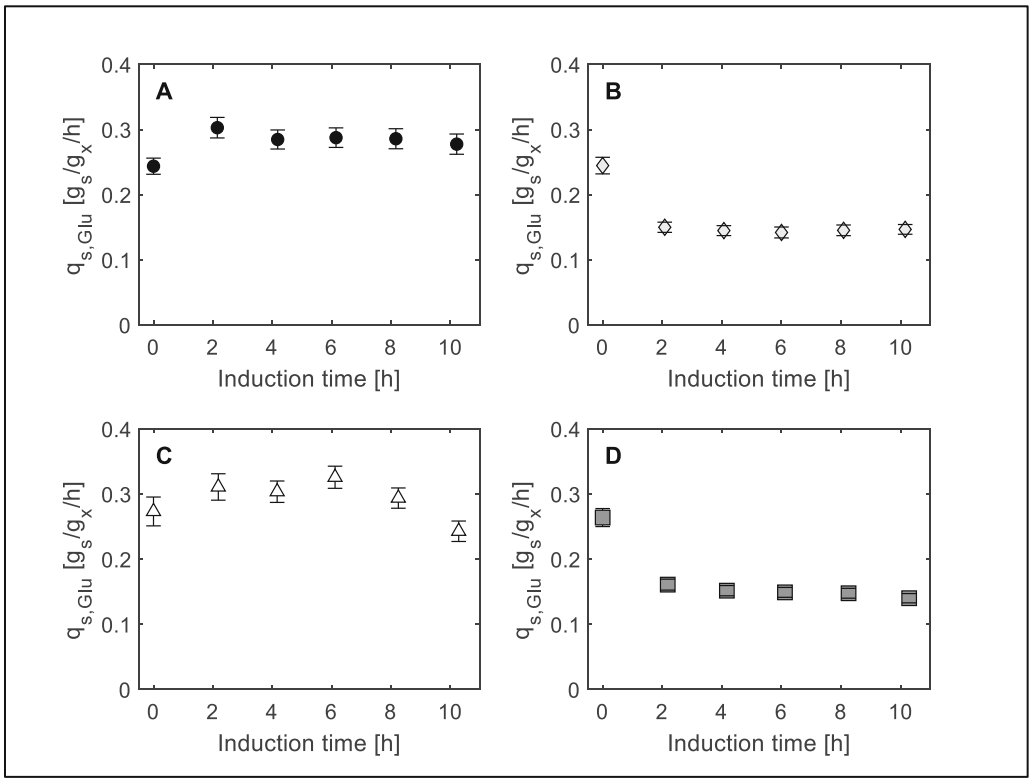
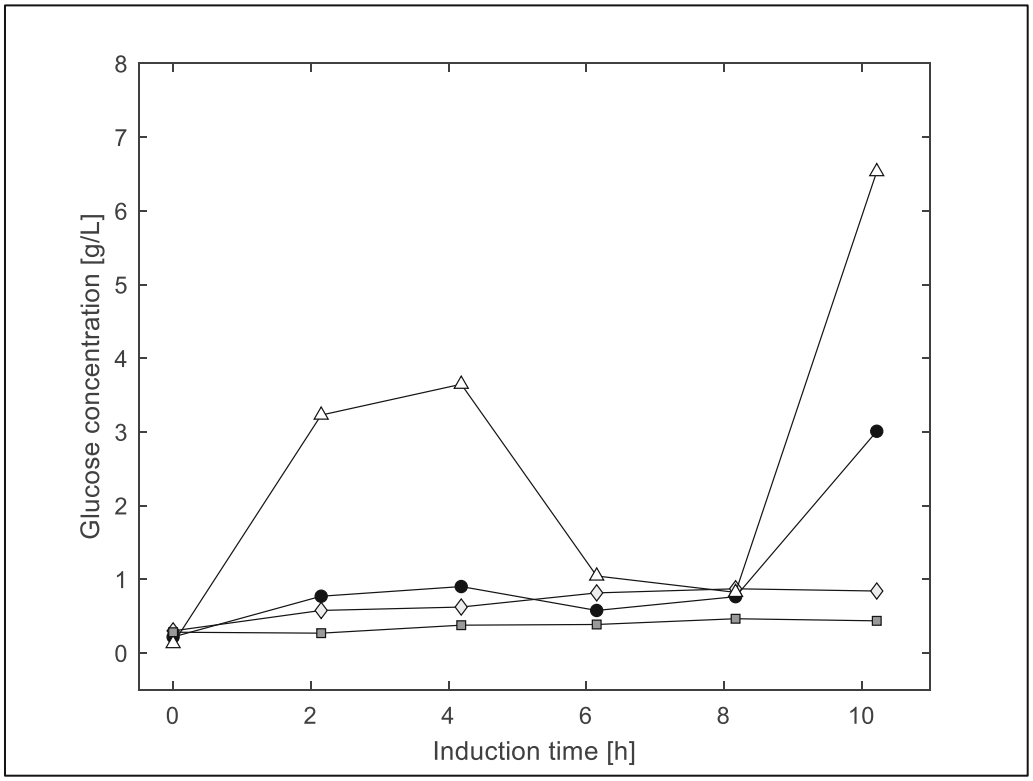


Figure 4: Specific glucose uptake rate of the induction timespan of IPTG-induced cultures.
A: μ_{ind} = 0.1 h^{-1}, T_{ind} = 30 °C. B: μ_{ind} = 0.05 h^{-1}, T_{ind} = 25 °C.
C: μ_{ind} = 0.1 h^{-1}, T_{ind} = 25 °C. D: μ_{ind} = 0.05 h^{-1}, T_{ind} = 30 °C.



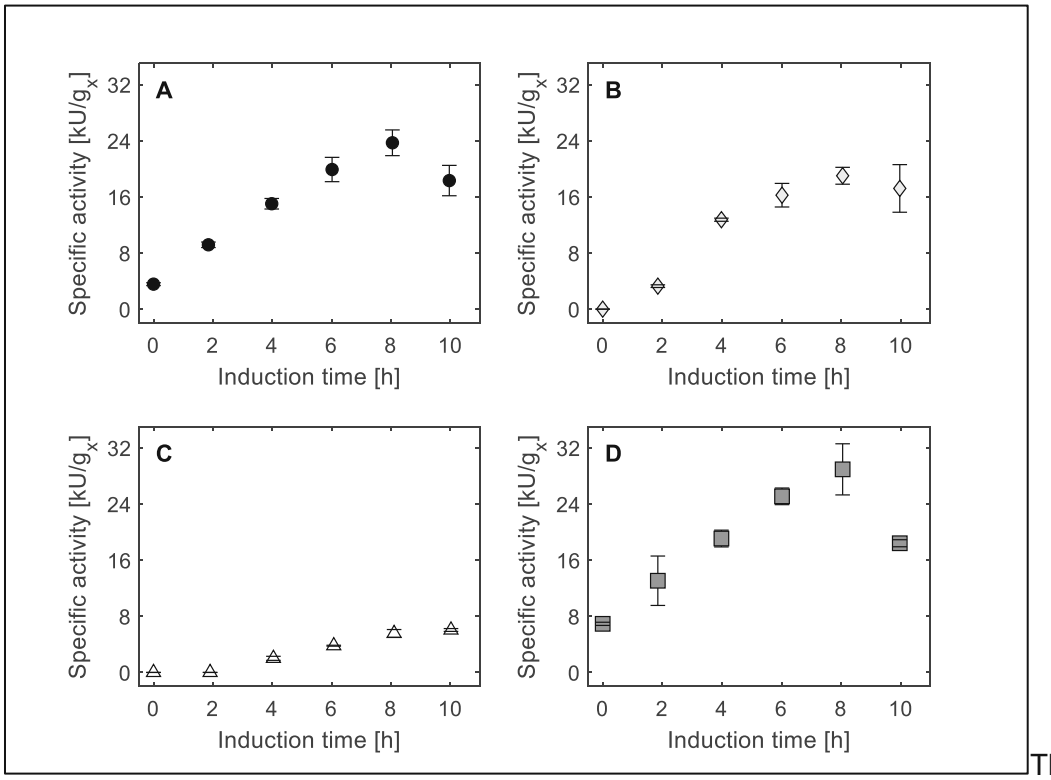
A high set specific substrate uptake rate and low induction temperature as applied in condition C seem to lead to stable productivity up to eight hours after induction, which is not the case with the other conditions. There, the productivity shows a sharp increase after two hours, followed by a decrease for the rest of the induction period.

Figure 5: Glucose concentration in the supernatant in the induction timespan of IPTG-induced cultures. Black circles: μ_{ind} = 0.1 h^{-1}, T_{ind} = 30 °C. Light grey diamonds: μ_{ind} = 0.05 h^{-1}, T_{ind} = 25 °C. White triangles: μ_{ind} = 0.1 h^{-1}, T_{ind} = 25 °C. Dark grey squares: μ_{ind} = 0.05 h^{-1}, T_{ind} = 30 °C.



In the second induction approach, rLTA expression was induced by pulsing lactose, followed immediately by a mixed feed of lactose and glucose, as explained above. The induction conditions are shown in Table 1. Lactose uptake as substrate was not considered when calculating the feed rate (Equation 2).

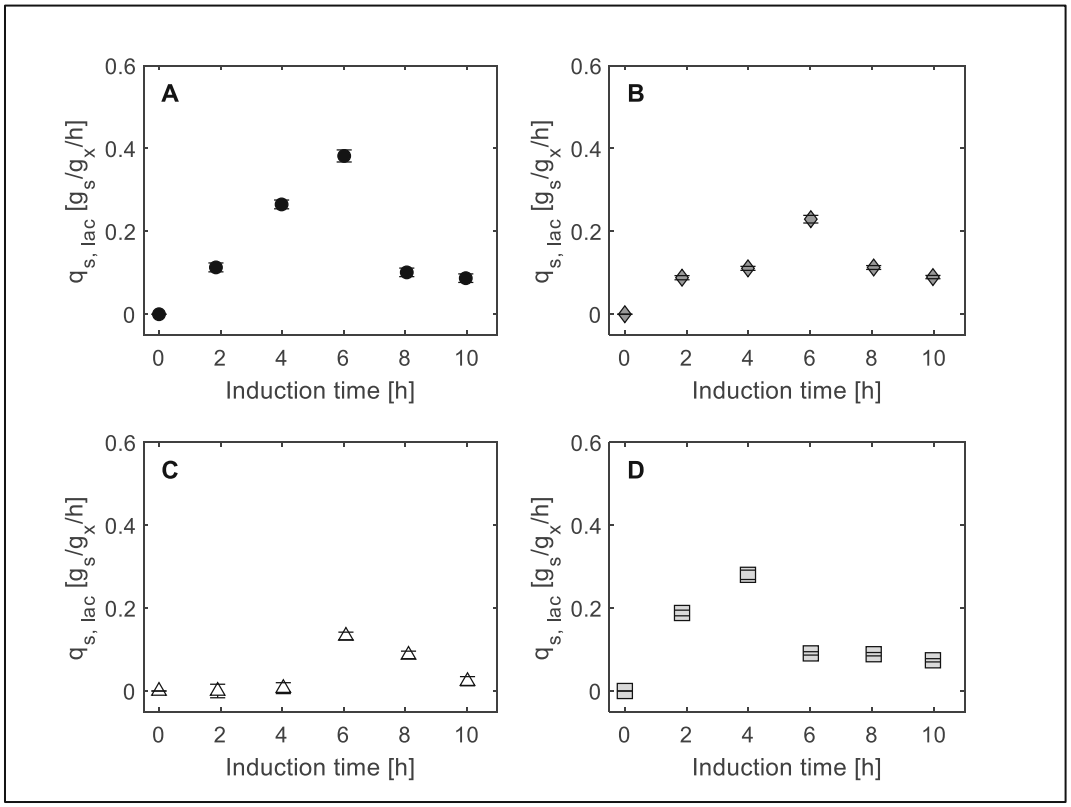
Figure 6: Biomass-specific rLTA activity of lactose-induced cultures in the induction timespan. A: μ_{ind} = 0.1 h^{-1}, T_{ind} = 30 °C. B: μ_{ind} = 0.05 h^{-1}, T_{ind} = 25 °C. C: μ_{ind} = 0.1 h^{-1}, T_{ind} = 25 °C. D: μ_{ind} = 0.05 h^{-1}, T_{ind} = 30 °C.



The curve of the specific activity of the fermentations with high induction temperature, conditions A and D respectively, has a similar trajectory (Figure 6). Firstly, upon induction, active enzyme seems to be expressed immediately.

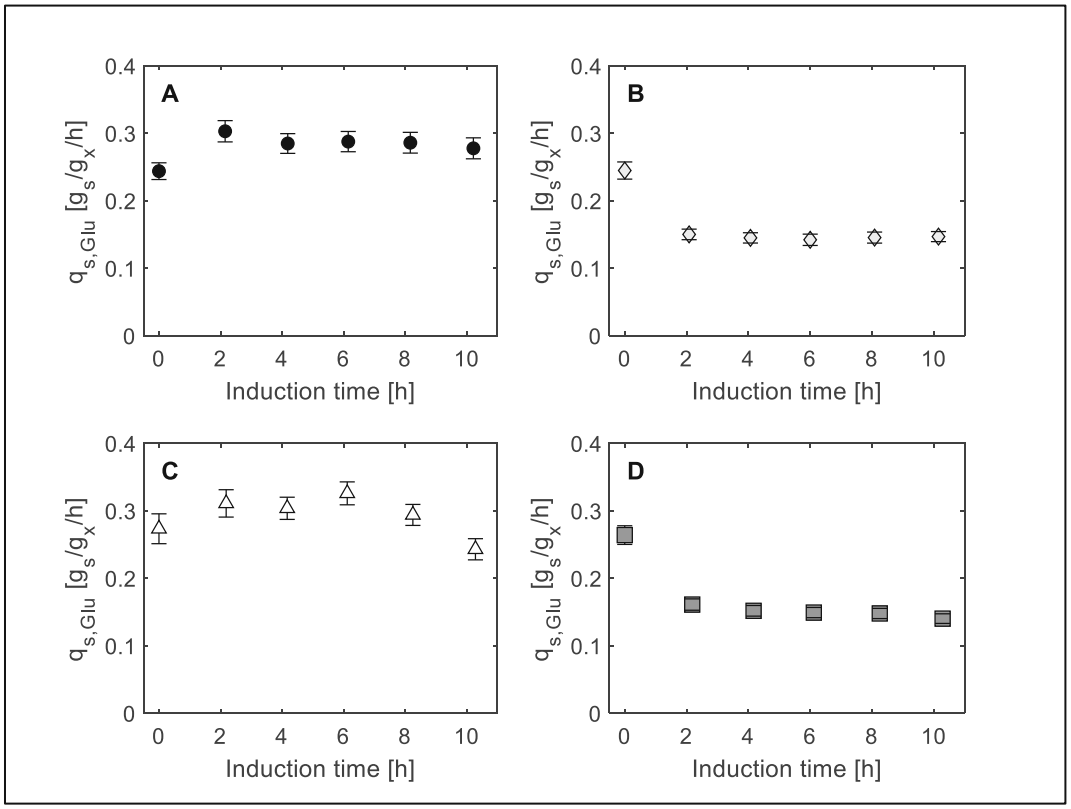
The curve of the specific activity of the fermentations with high induction temperature, conditions A and D respectively, has a similar trajectory (Figure 6). Firstly, upon induction, active enzyme seems to be expressed immediately.

Figure 7: Specific lactose uptake rate of the induction timespan of lactose-induced cultures.
A: μ_{ind} = 0.1 h^{-1}, T_{ind} = 30 °C. B: μ_{ind} = 0.05 h^{-1}, T_{ind} = 25 °C.
C: μ_{ind} = 0.1 h^{-1}, T_{ind} = 25 °C. D: μ_{ind} = 0.05 h^{-1}, T_{ind} = 30 °C.



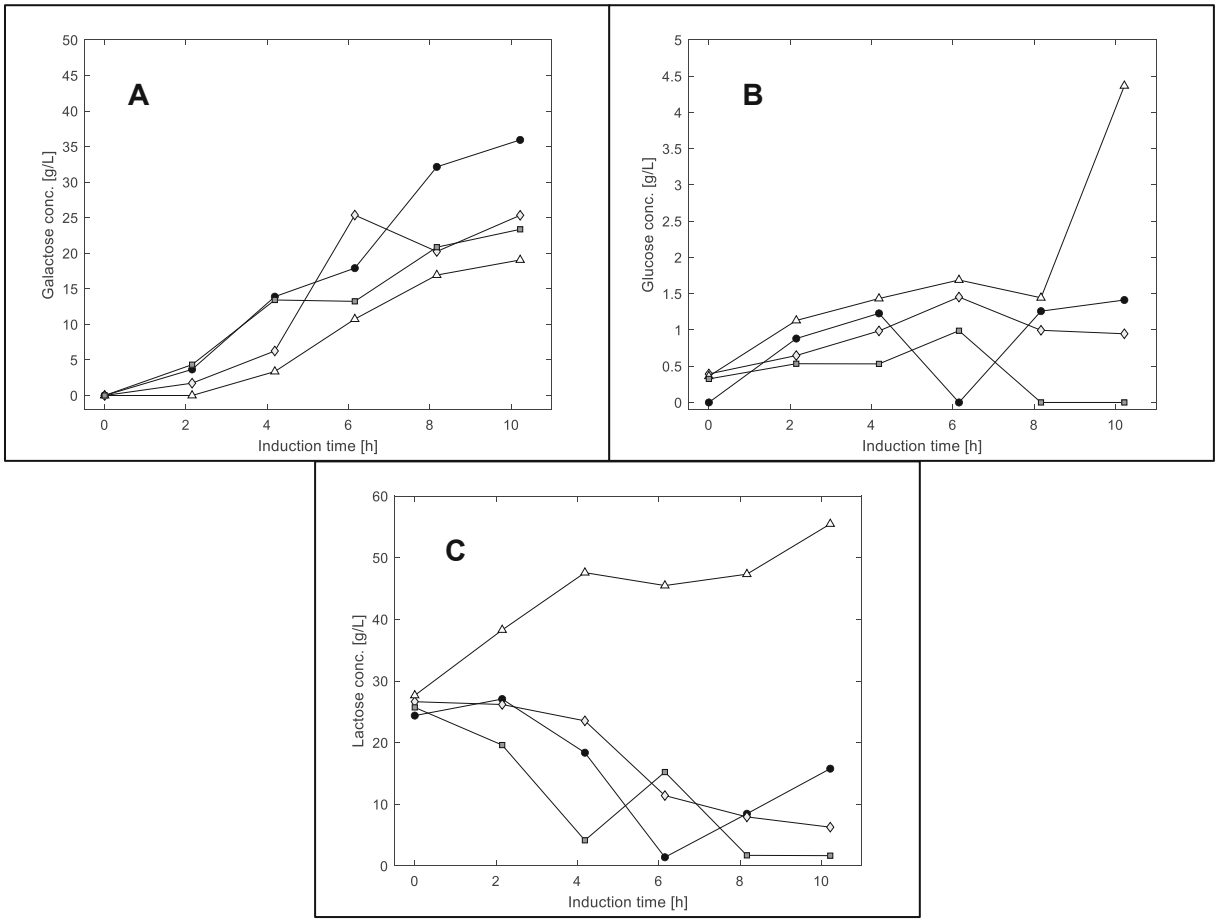
At the end of the fed-batch phase, where glucose is fed at a higher but still substrate-limiting rate, a lactose adaptation pulse was performed to ensure that enough lactose is able to enter the cell to derepress the lac operon and thus initiate the lactose uptake machinery. From this point, the substrate feed rate setpoint is substantially lower than in the fed batch, allowing lactose to be taken up concomitantly with glucose during the induced fed batch.

Figure 8: : Specific glucose uptake rate of the induction timespan of lactose-induced cultures. A: μ_{ind} = 0.1 h^{-1}, T_{ind} = 30 °C. B: μ_{ind} = 0.05 h^{-1}, T_{ind} = 25 °C. C: μ_{ind} = 0.1 h^{-1}, T_{ind} = 25 °C. D: μ_{ind} = 0.05 h^{-1}, T_{ind} = 30 °C.



At an induction temperature of 30 °C, lactose uptake increases at the beginning of the induction time span (Figure 7). Thus, enough inducer seems to be present inside the cell to bind to the highly expressed lac repressor protein and thus induce rLTA production. The specific lactose uptake rate decreases drastically after six to eight hours of induction for all fermentations. When looking at glucose accumulation in the cultivation media, all fermentation except for condition A exhibit glucose accumulation peaking at six hours of induction. This could be the reason for the drop in the lactose uptake rate, since glucose inhibits lactose permease and thus lactose uptake as well as transcription of the lac operon. [51] Since lactose permease is inhibited by glucose uptake, there may not be enough lactose present in the cell to titrate LacI to derepress the T5 promoter, which might explain the drop in productivity after six hours of induction. Condition C on the other hand (μ =0.05 h^{-1}, T_{ind} = 25 °C) displays overfeeding of lactose, as the lactose uptake rate only slightly rises after 6 h of induction, and lactose accumulates throughout the whole induction period (**Fehler! Verweisquelle konnte nicht gefunden werden.**).

Figure 9: Sugar concentrations of lactose-induced cultures in the supernatant during the induction timespan. A: Galactose concentration. B: Glucose concentration. C: Lactose concentration. Black circles: μ_{ind} = 0.1 h^{-1}, T_{ind} = 30 °C. Light grey diamonds: μ_{ind} = 0.05 h^{-1}, T_{ind} = 25 °C. White triangles: μ_{ind} = 0.1 h^{-1}, T_{ind} = 25 °C. Dark grey squares: μ_{ind} = 0.05 h^{-1}, T_{ind} = 30 °C.



Additionally, at the end of the induction timespan, glucose accumulates drastically in the broth, indicating overfeeding, which is also shown in the drop of $q_{s,Glu}$, presumably due to downregulation of energy metabolism because of lower temperature. [20] Although there is glucose accumulating in the broth with the other induction conditions as well, $q_{s,Glu}$ remains constant from 2 h to 10 h after induction.

When looking at condition B (low μ, low T), productivity rises within 4 hours. Then, the specific activity remains stable for 6 hours. The achieved plateau of specific activity is lower than the maximal specific activities from the fermentations with 30 °C induction temperature, rendering this approach less favorable.

The C-balances for all fermentations displayed in Table 3 and Table 4 indicate that every carbon from substrate is roughly accounted for. For both inducers, low specific growth rate and high temperature (μ_{ind} = 0.05 h^{-1}, T_{ind} = 30 °C) seem to yield the most favorable results concerning active enzyme expression. However, with IPTG as an inducer, productivity surges only 2h after the induction pulse, whereas with lactose the productivity shows a constant increase until 8 h after the induction pulse and inducer feed start.

This may be explained due to the fact that with IPTG, inducer is only pulsed once and not fed continuously. [36] After growing to a certain cell density, the pulsed IPTG may not be enough to titrate all present LacI molecules. This, in turn, reduces recombinant protein expression and thus the metabolic burden on the cell [52], with the consequence that the relative target gene expression seems not to decrease during the induction phase (supplementary material,

Table 2). A negative effect on target gene expression occurs with lactose induction. Here, a relative decrease can be seen over time. Since the biomass yield $Y_{x/s}$ increases substantially in the timespan between 2 and 10 hours of induction (Table 5 and Table 6), the metabolic burden on the cell by target protein production is reduced. This could be explained by promoter deletion as hypothesized by Kawe et al [35]. Another reason could be that not enough ampicillin is present in the media. Since the resistance mechanism relies on Ampicillin degradation by beta-lactamase, the pulsed amount in the beginning of the fermentation might not be enough to maintain the selection pressure. Thus, a mixed population of plasmid-bearing strains as well as non-plasmid bearing strains could be present by the end of the induction timespan due to insufficient amount of antibiotics in the media. [1,[53]

Conclusion & Outlook

It can be concluded that (I) the type of inducer, (II) the substrate feed rate during induction as well as (III) the induction temperature seem to influence rLTA production in the described expression system. We showed that by using lactose as inducer, specific rLTA production rates can be prolonged in *E. coli* BL21 fed batch cultivations employing the T5 promoter system. We can summarize that the following parameters yield the most favorable results with respect to active rLTA production and should be taken into consideration in future induced fed-batch fermentation strategies using this particular expression system:

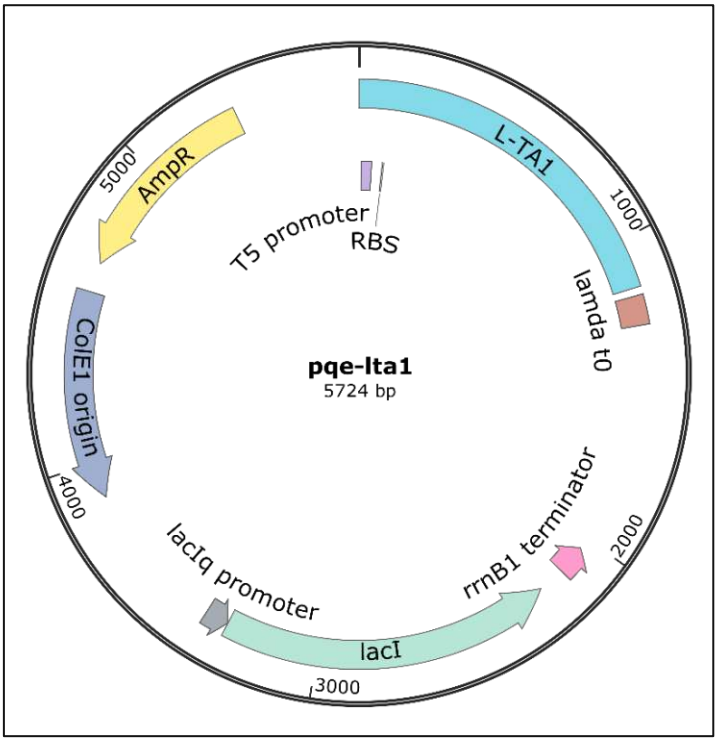
- μ_{ind} = 0.05 h^{-1} & T_{ind} = 30 °C
- Lactose induction for 6-8 h.

The use of glycerol instead of glucose might lead to an improvement, since the carbon catabolite repression mechanism for this particular expression system is defined by the highly expressed LacI. [34] Furthermore, in order to counter a possible effect of plasmid loss, ampicillin might be pulsed to ensure plasmid stability [36] or a different strategy for selection should be investigated to increase productivity and consistency.

Supplementary material

Expression vector

Figure 10: The plasmid pQE-LTA1 was constructed by on the pQE-80L backbone (Qiagen, Hilden, Germany). AmpR: gene encoding ß-Lactamase for ampicillin resistance. RBS: Ribosome binding site on mRNA. ColE1 origin: origin of replication. L-TA1: gene encoding L-Threonine aldolase. lacI: gene encoding LacI repressor.



Influence of induction parameters on inclusion body formation

For the investigation of inclusion bodies, the insoluble fraction of lysates with BugBuster Mastermix were washed with deionized water and resuspended in 50 µL 2x Laemmli buffer. Then, they were subjected to heating at 95 °C for 10 min and subsequently centrifuged. The supernatant was loaded on a polyacrylamide gel and SDS-PAGE was performed at 160 V for 40 min.

To investigate the hypothesis whether the amount of insoluble protein is influenced by the type of inducer, induction temperature and induction feed rate, the insoluble lysate fraction of end-of-fermentation sample pellets was separated on a polyacrylamide gel. The results are shown in Figure 11 for IPTG induction and in Figure 12 for lactose induction respectively.

It can be seen that only a small percentage of the insoluble fraction seems to contain monomeric rLTA (37.98 kDa), presumably. A substantially more prominent band just above possibly coincides with the monomer of the lac repressor protein (38.7 kDa), which could be explained by 10-fold higher expression due to the *lacI*^{q} promoter. This high expression rate may in turn lead to aggregation of insoluble inclusion bodies of lac repressor. Since no basal expression of rLTA can be seen in the samples prior to induction according to the activity

assay, enough LacI seems to fold properly to ensure tight repression when no inducer is present.

Figure 11: SDS-Page of the insoluble fraction of the end-of-fermentation pellets of IPTG induced fermentations. The leftmost lane is the ladder. The run order is described in the table.

Run	Conditions	% L-TA1 (presumably)
A	μ = 0.1 T = 30 °C	4.88
B	μ = 0.05 T = 25 °C	3.48
C	μ = 0.1 T = 25 °C	2.86
D	μ = 0.05 T = 30 °C	6.69

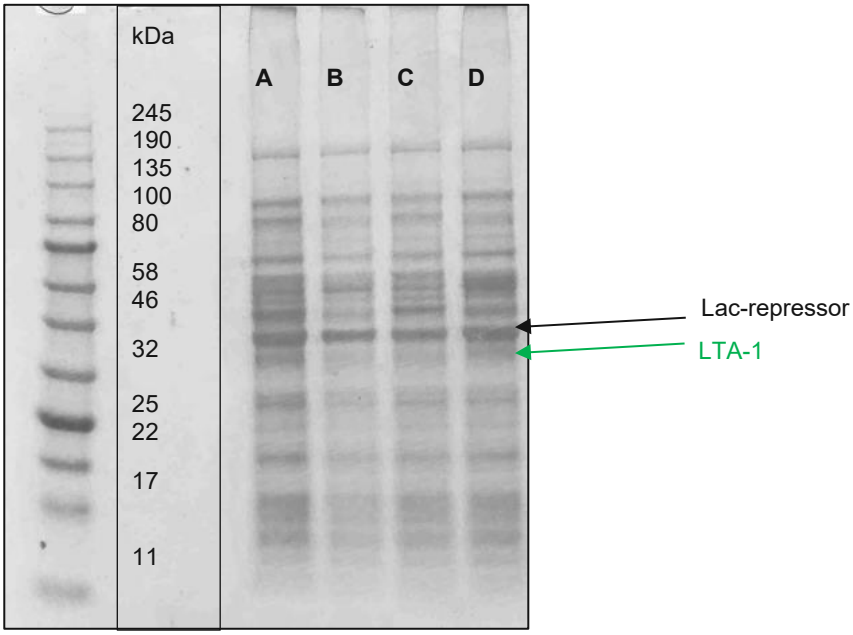
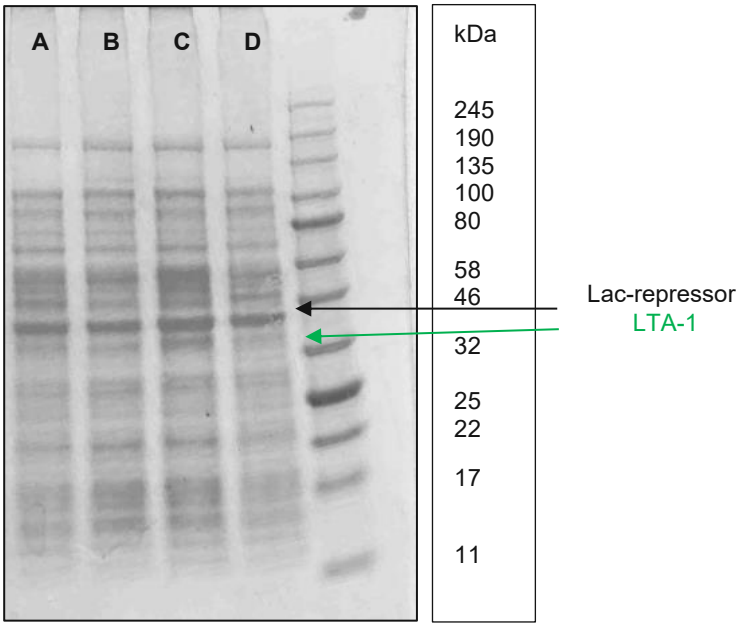


Figure 12: SDS-Page of the insoluble fraction of the end-of-fermentation pellets of lactose induced fermentations. The leftmost lane is the ladder. The run order is described in the table.

Run	Conditions	% L-TA1 (presumably)
A	μ = 0.1 T = 30 °C	2.40
B	μ = 0.05 T = 25 °C	3.67
C	μ = 0.1 T = 25 °C	7.15
D	μ = 0.05 T = 30 °C	4.28



Relative expression ratios of T5 promoter

To determine the change in target gene expression over time, samples from 2 h and 10 h after induction of condition D (low μ, high T) with both inducers were used for TaqMan qPCR assays. The measurements in triplicates were performed by Microsynth AG (Switzerland). As target

sequence, the T5 promoter, which controls expression of rLTA, was used. The d-1-deoxyxylulose 5-phosphate synthase gene (*dxs*) was used as chromosomal single copy reference housekeeping gene. [54] To calculate the relative target gene expression ratios between the time points of the respective inductions, the $2^{-\Delta\Delta Ct}$ method was used. [55] With equations 3 and 4, the relative change in gene expression can be estimated using the respective threshold cycle values. The results are shown in

Table 2.

$$Ratio = 2^{-\Delta\Delta C_t}$$

Equation 3: Relative gene expression ratio calculation

$$\Delta\Delta C_t = \left(C_{t,T5,10h} - C_{t,dxs,10h}\right) - \left(C_{t,T5,2h} - C_{t,dxs,2h}\right)$$

Equation 4: Calculation of the $\Delta\Delta C_t$ value

$C_{t,T5,10h}$	……..	Threshold cycle for target sequence from sample 10 h after induction
$C_{t,dxs,10h}$	……..	Threshold cycle for reference sequence from sample 10 h after induction
$C_{t,T5,2h}$	……..	Threshold cycle for target sequence from sample 2 h after induction
$C_{t,dxs,2h}$	……..	Threshold cycle for reference sequence from sample 2 h after induction

Table 2: Change in T5 promoter sequence expression from 2 h to 10 h after induction calculated with the $2^{-\Delta\Delta Ct}$ method

Sample	Mean fold change in gene expression $2^{-\Delta\Delta Ct}$
IPTG 2 h induced	0.98 ± 0.09
IPTG 10 h induced	
Lactose 2 h induced	0.31 ± 0.02
Lactose 10 h induced	

For IPTG induction, the change in gene expression seemed to have remained at the same level throughout the measured period. Lactose induction, however, seems to lead to a 3-fold reduction of T5-promoter-regulated gene expression in the investigated time frame.

Carbon Balances

Table 3: C-balances of IPTG-induced fermentations in the induction timespan
A: μ_{ind} = 0.1 h^{-1}, T_{ind} = 30 °C. B: μ_{ind} = 0.05 h^{-1}, T_{ind} = 25 °C.
C: μ_{ind} = 0.1 h^{-1}, T_{ind} = 25 °C. D: μ_{ind} = 0.05 h^{-1}, T_{ind} = 30 °C.

Induction Time [h]	A	B	C	D
0	0.94 ± 0.26	0.93 ± 0.26	1.09 ± 0.45	0.89 ± 0.24
2	0.91 ± 0.06	0.72 ± 0.24	1.02 ± 0.24	0.92 ± 0.22
4	0.95 ± 0.06	1.06 ± 0.14	0.98 ± 0.09	0.99 ± 0.11
6	0.85 ± 0.09	0.77 ± 0.28	0.85 ± 0.10	0.77 ± 0.08
8	0.94 ± 0.10	1.00 ± 0.23	0.93 ± 0.09	0.93 ± 0.08
10	0.89 ± 0.09	1.03 ± 0.1	0.94 ± 0.10	0.99 ± 0.10

Table 4: C-balances of lactose-induced fermentations in the induction timespan
A: μ_{ind} = 0.1 h^{-1}, T_{ind} = 30 °C. B: μ_{ind} = 0.05 h^{-1}, T_{ind} = 25 °C.
C: μ_{ind} = 0.1 h^{-1}, T_{ind} = 25 °C. D: μ_{ind} = 0.05 h^{-1}, T_{ind} = 30 °C.

Induction Time [h]	A	B	C	D
0	-0.01 ± 0.15	0.14 ± 0.1	-0.15 ± 0.18	0.23 ± 0.07
2	1.10 ± 0.10	1.01 ± 0.1	1.49 ± 0.12	1.28 ± 0.09
4	0.79 ± 0.07	1.16 ± 0.1	1.26 ± 0.14	0.8 ± 0.07
6	0.70 ± 0.07	0.85 ± 0.1	1.14 ± 0.11	1.21 ± 0.11
8	1.20 ± 0.1	1.11 ± 0.13	0.98 ± 0.12	0.93 ± 0.13
10	1.20 ± 0.07	1.16 ± 0.13	1.21 ± 0.12	1.19 ± 0.18

Yields

Table 5: Yield coefficients $Y_{X/S}$ [$cmol_X/cmol_S$] of IPTG-induced fermentations in the induction timespan
A: μ_{ind} = 0.1 h^{-1}, T_{ind} = 30 °C. B: μ_{ind} = 0.05 h^{-1}, T_{ind} = 25 °C.
C: μ_{ind} = 0.1 h^{-1}, T_{ind} = 25 °C. D: μ_{ind} = 0.05 h^{-1}, T_{ind} = 30 °C.

Induction Time [h]	A	B	C	D
0	0.45 ± 0.26	0.45 ± 0.26	0.65 ± 0.44	0.42 ± 0.24
2	0.43 ± 0.05	0.53 ± 0.22	0.58 ± 0.21	0.52 ± 0.19
4	0.41 ± 0.03	0.44 ± 0.03	0.43 ± 0.04	0.43 ± 0.03
6	0.47 ± 0.05	0.46 ± 0.05	0.41 ± 0.03	0.42 ± 0.03
8	0.52 ± 0.05	0.57 ± 0.08	0.47 ± 0.05	0.43 ± 0.04
10	0.61 ± 0.06	0.66 ± 0.06	0.59 ± 0.07	0.47 ± 0.05

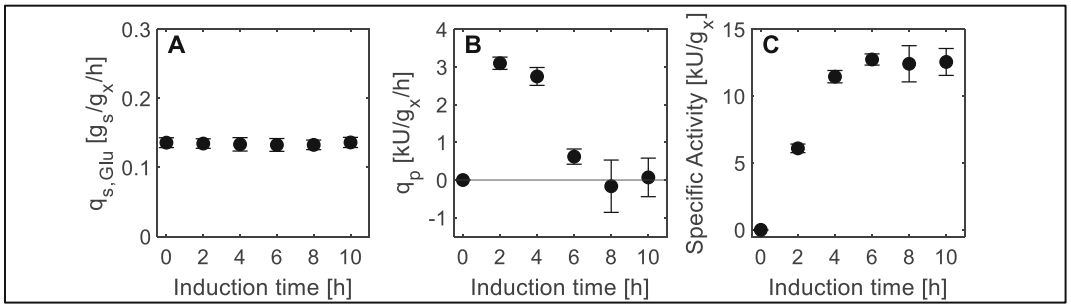
Table 6: Yield coefficients $Y_{X/S}$ [$cmol_x/cmol_s$] of lactose-induced fermentations in the induction timespan A: μ_{ind} = 0.1 h^{-1}, T_{ind} = 30 °C. B: μ_{ind} = 0.05 h^{-1}, T_{ind} = 25 °C. C: μ_{ind} = 0.1 h^{-1}, T_{ind} = 25 °C. D: μ_{ind} = 0.05 h^{-1}, T_{ind} = 30 °C.

Induction Time [h]	A	B	C	D
0	-0.20 ± 0.02	-0.14 ± 0.02	0.08 ± 0.01	-0.07 ± 0.01
2	0.46 ± 0.09	0.43 ± 0.07	0.61 ± 0.09	0.48 ± 0.07
4	0.45 ± 0.06	0.48 ± 0.05	0.50 ± 0.06	0.43 ± 0.03
6	0.42 ± 0.06	0.38 ± 0.03	0.44 ± 0.05	0.61 ± 0.05
8	0.69 ± 0.08	0.51 ± 0.05	0.52 ± 0.07	0.50 ± 0.04
10	0.67 ± 0.04	0.57 ± 0.07	0.71 ± 0.04	0.61 ± 0.06

Scale-up experiment

A scale-up experiment was performed using a Sartorius C bioreactor with a working volume of 20 L and a batch volume of 6 L. IPTG as inducer with induction condition D, with a specific biomass growth rate of μ = 0.05 h^{-1} and temperature of T_{ind} = 30 °C, was chosen for the scale-up. The culture conditions and parameters for the batch and uninduced fed-batch as well as the sampling times and the sample analytics were the same as for the corresponding small-scale experiment. The resulting specific glucose uptake rate, productivity as well as specific activity can be seen in Figure 13.

Figure 13: Scale-up experiment with μ = 0.05 h^{-1} and T_{ind} = 30 °C. A: Specific glucose uptake rate. B: Productivity. C: Specific rLTA Activity.



The specific substrate uptake rate remained constant during the induced fed-batch phase, as the glucose concentration could not be measured due to a faulty HPLC, thus it was assumed that all glucose which was fed was subsequently consumed completely. When comparing the productivity as well as the specific enzyme activity to the corresponding small-scale experiment, the productivity q_p was highest at the beginning of the induction timespan, followed by a drastic decrease. A similar trend can be observed at the small-scale experiment, although there the productivity peaks after two hours followed by a sharp decrease. The peak productivity values, calculated from the specific enzyme activity, are

overall lower than in the corresponding small-scale experiment with the same induction conditions. This could be attributed to effects caused by scaling up the bioprocess, which include differences in mixing, oxygenation and viscosity. [56–58]

Table 7: Yield coefficient $Y_{X/S}$ [$cmol_x/cmol_s$] and C-balance for the scale-up experiment.

Induction Time [h]	$Y_{X/S}$ [$cmol_x/cmol_s$]	C-balance [ ]
0	0.48 ± 0.03	0.96 ± 0.20
2	0.48 ± 0.15	1.00 ± 0.15
4	0.50 ± 0.11	0.99 ± 0.11
6	0.48 ± 0.46	0.98 ± 0.47
8	0.50 ± 0.46	0.94 ± 0.53
10	0.42 ± 0.14	0.93 ± 0.15

References

[1] J.W. Dubendorf, F.W. Studier, Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with lac repressor, J. Mol. Biol. 219 (1991) 45–59. https://doi.org/10.1016/0022-2836(91)90856-2.

[2] F.W. Studier, Protein production by auto-induction in high density shaking cultures., Protein Expr. Purif. 41 (2005) 207–234. https://doi.org/10.1016/j.pep.2005.01.016.

[3] P.G. Blommel, K.J. Becker, P. Duvnjak, B.G. Fox, Enhanced bacterial protein expression during auto-induction obtained by alteration of lac repressor dosage and medium composition., Biotechnol. Prog. 23 (2007) 585–98. https://doi.org/10.1021/bp070011x.

[4] T. Sano, C.R. Cantor, Expression of a cloned streptavidin gene in Escherichia coli, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 142–146. https://doi.org/10.1073/pnas.87.1.142.

[5] D.J. Wurm, J. Hausjell, S. Ulonska, C. Herwig, O. Spadiut, Mechanistic platform knowledge of concomitant sugar uptake in Escherichia coli BL21(DE3) strains., Sci. Rep. 7 (2017) 45072. https://doi.org/10.1038/srep45072.

[6] J. Hausjell, J. Weissensteiner, C. Molitor, H. Halbwirth, O. Spadiut, E. coli HMS174(DE3) is a sustainable alternative to BL21(DE3), Microb. Cell Fact. 17 (2018) 169. https://doi.org/10.1186/s12934-018-1016-6.

[7] F.W. Studier, Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system, J. Mol. Biol. 219 (1991) 37–44. https://doi.org/10.1016/0022-2836(91)90855-Z.

[8] P.G. Blommel, K.J. Becker, P. Duvnjak, B.G. Fox, Enhanced Bacterial Protein Expression During Auto-Induction Obtained by Alteration of Lac Repressor Dosage and Medium Composition, Biotechnol. Prog. 23 (2008) 585–598. https://doi.org/10.1021/bp070011x.

[9] M. Kawe, U. Horn, A. Plückthun, Facile promoter deletion in Escherichia coli in response to leaky expression of very robust and benign proteins from common expression vectors, Microb. Cell Fact. 8 (2009) 8. https://doi.org/10.1186/1475-2859-8-8.

[10] L. Vidal, P. Ferrer, G. Álvaro, M.D. Benaiges, G. Caminal, Influence of induction and operation mode on recombinant rhamnulose 1-phosphate aldolase production by Escherichia coli using the T5 promoter, J. Biotechnol. 118 (2005) 75–87.

https://doi.org/10.1016/j.jbiotec.2005.02.012.

[11] A. Schuller, M. Cserjan-Puschmann, C. Tauer, J. Jarmer, M. Wagenknecht, D. Reinisch, R. Grabherr, G. Striedner, Escherichia coli σ 70 promoters allow expression rate control at the cellular level in genome-integrated expression systems, (n.d.). https://doi.org/10.1186/s12934-020-01311-6.

[12] R. Gentz, H. Bujard, Promoters recognized by Escherichia coli RNA polymerase selected by function: Highly efficient promoters from bacteriophage T5, J. Bacteriol. 164 (1985) 70–77. https://doi.org/10.1128/jb.164.1.70-77.1985.

[13] M.P. Calos, DNA sequence for a low-level promoter of the lac repressor gene and an “up” promoter mutation, Nature. 274 (1978) 762–765. https://doi.org/10.1038/274762a0.

[14] G.L. Rosano, E.A. Ceccarelli, Recombinant protein expression in Escherichia coli: Advances and challenges, Front. Microbiol. 5 (2014). https://doi.org/10.3389/fmicb.2014.00172.

[15] Patent Evaluation Biologicals , Expert Opin. Ther. Pat. 6 (1996) 789–793. https://doi.org/10.1517/13543776.6.8.789.

[16] F. Saida, M. Uzan, B. Odaert, F. Bontems, Expression of Highly Toxic Genes in E. coli: Special Strategies and Genetic Tools, Curr. Protein Pept. Sci. 7 (2006) 47–56. https://doi.org/10.2174/138920306775474095.

[17] J. Pinsach, C. de Mas, J. López-Santín, Induction strategies in fed-batch cultures for recombinant protein production in Escherichia coli: Application to rhamnulose 1-phosphate aldolase, Biochem. Eng. J. 41 (2008) 181–187. https://doi.org/10.1016/j.bej.2008.04.013.

[18] RCSB PDB - 5VYE: Crystal Structure of L-Threonine Aldolase from Pseudomonas putida, (n.d.).

[19] J.Q. Liu, S. Ito, T. Dairi, N. Itoh, M. Kataoka, S. Shimizu, H. Yamada, Gene cloning, nucleotide sequencing, and purification and characterization of the low-specificity L-threonine aldolase from Pseudomonas sp. strain NCIMB 10558, Appl. Environ. Microbiol. 64 (1998) 549–554. https://doi.org/10.1128/aem.64.2.549-554.1998.

[20] cis-Repressed pQE Vector Set - QIAGEN Online Shop, (n.d.).

[21] C.T. Chung, R.H. Miller, Preparation and Storage of Competent Escherichia coli Cells, Methods Enzymol. 218 (1993) 621–627. https://doi.org/10.1016/0076-6879(93)18045-E.

[22] M.P. DeLisa, J. Li, G. Rao, W.A. Weigand, W.E. Bentley, Monitoring GFP-operon fusion protein expression during high cell density cultivation ofEscherichia coli using an on-line optical sensor, Biotechnol. Bioeng. 65 (1999) 54–64. https://doi.org/10.1002/(SICI)1097-0290(19991005)65:1<54::AID-BIT7>3.0.CO;2-R.

[23] Q.J. (TU Wien), DIPLOMARBEIT Expression tuning in Escherichia coli via a glucose / lactose mixed feed system, (n.d.) 1–86.

[24] K. Fesko, G.A. Strohmeier, R. Breinbauer, Expanding the threonine aldolase toolbox for the asymmetric synthesis of tertiary a-amino acids, Appl. Microbiol. Biotechnol. 99 (2015) 9651–9661. https://doi.org/10.1007/s00253-015-6803-y.

[25] M. Lanzer, H. Bujard, Promoters largely determine the efficiency of repressor action, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 8973–8977. https://doi.org/10.1073/pnas.85.23.8973.

[26] A. Fernández-Castané, C.E. Vine, G. Caminal, J. López-Santín, Evidencing the role of lactose permease in IPTG uptake by Escherichia coli in fed-batch high cell density cultures, J. Biotechnol. 157 (2012) 391–398. https://doi.org/10.1016/j.jbiotec.2011.12.007.

[27] M. Gadgil, V. Kapur, W.S. Hu, Transcriptional response of Escherichia coli to temperature shift, Biotechnol. Prog. 21 (2005) 689–699. https://doi.org/10.1021/bp049630l.

[28] B. Görke, J. Stülke, Carbon catabolite repression in bacteria: Many ways to make the most out of nutrients, Nat. Rev. Microbiol. 6 (2008) 613–624. https://doi.org/10.1038/nrmicro1932.

[29] D.S.W. Ow, P.M. Nissom, R. Philp, S.K.W. Oh, M.G.S. Yap, Global transcriptional analysis of metabolic burden due to plasmid maintenance in Escherichia coli DH5α during batch fermentation, Enzyme Microb. Technol. 39 (2006) 391–398. https://doi.org/10.1016/j.enzmictec.2005.11.048.

[30] A. Kremling, J. Geiselmann, D. Ropers, H. de Jong, Understanding carbon catabolite repression in Escherichia coli using quantitative models, Trends Microbiol. 23 (2015) 99–109. https://doi.org/10.1016/j.tim.2014.11.002.

[31] R. Grabherr, E. Nilsson, G. Striedner, K. Bayer, Stabilizing plasmid copy number to improve recombinant protein production, Biotechnol. Bioeng. 77 (2002) 142–147. https://doi.org/10.1002/bit.10104.

[32] T. Korpimäki, J. Kurittu, M. Karp, Surprisingly fast disappearance of β-lactam selection

pressure in cultivation as detected with novel biosensing approaches, J. Microbiol. Methods. 53 (2003) 37–42. https://doi.org/10.1016/S0167-7012(02)00213-0.

[33] C. Lee, J. Kim, S.G. Shin, S. Hwang, Absolute and relative QPCR quantification of plasmid copy number in Escherichia coli, J. Biotechnol. 123 (2006) 273–280. https://doi.org/10.1016/j.jbiotec.2005.11.014.

[34] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method, Methods. 25 (2001) 402–408. https://doi.org/10.1006/meth.2001.1262.

[35] J. Li, J. Jaitzig, P. Lu, R.D. Süssmuth, P. Neubauer, Scale-up bioprocess development for production of the antibiotic valinomycin in Escherichia coli based on consistent fed-batch cultivations, Microb. Cell Fact. 14 (2015) 83. https://doi.org/10.1186/S12934-015-0272-Y.

[36] B. Li, M. Sha, Scale-Up of Escherichia coli Fermentation from Small Scale to Pilot Scale Using Eppendorf Fermentation Systems, (2016).

[37] P. Neubauer, S. Junne, Scale-down simulators for metabolic analysis of large-scale bioprocesses, Curr. Opin. Biotechnol. 21 (2010) 114–121. https://doi.org/10.1016/J.COPBIO.2010.02.001.

Arbeitsbericht: Innovationsscheck mit Selbstbehalt

Valanx Biotech GmbH

Opernring 16/1

8010 Graz

Forschungsgruppe Integrierte Bioprozessentwicklung

Institut für Verfahrenstechnik, Umwelttechnik und Technische Biowissenschaften

TU Wien

Getreidemarkt 9/166

1060 Wien

Im folgenden Arbeitsbericht wird die Zusammenarbeit von Valanx Biotech GmbH und der Forschungsgruppe Integrierte Bioprozessentwicklung des Instituts für Verfahrenstechnik, Umwelttechnik und Technische Biowissenschaften der TU Wien im Zuge des Innovationsschecks mit Selbstbehalt im Zeitraum August bis November 2019 beschrieben.

Projektass. Dr.techn. Johanna Hausjell

Associate Prof. Dipl.-Ing. Dr.nat.techn. Oliver Spadiut

Maximilian Winkler, BSc

Michael Lukesch, MSc

Ausgangslage und Zielsetzung

Das aus *Pseudomonas putida* stammende Enzym L-Threonin Aldolase [12–14] (LTA-1, UniProtKB O50584) ist eine 37 kDa große, als Homotetramer aufgebaute Aldolase mit niedriger Substratspezifität, die Pyridoxal-5-Phosphat als Cofaktor benötigt. LTA-1 katalysiert unter anderem die Spaltung von DL-Phenylserin zu Benzaldehyd und Glycin. Es ist jedoch ebenso fähig, die Reaktion von Bicyclo[2.2.1]hept-5-en-2-carbaldehyd und Glycin zu einer nichtkanonischen Aminosäure zu katalysieren (Abbildung 1). Das Patent für diese neuartige Aminosäure, Norl, sowie der Syntheseweg über LTA-1 wurde von Valanx Biotech GmbH (im Folgenden als Valanx bezeichnet) angemeldet (Pat. No. WO2019016354).

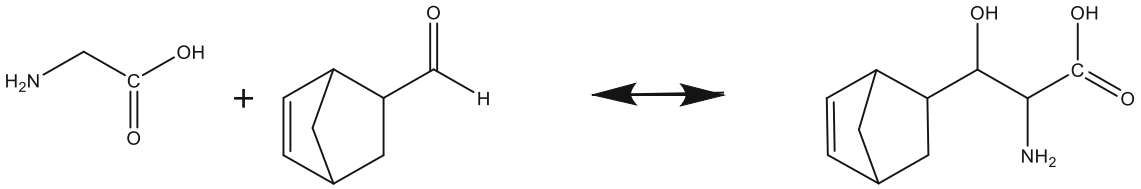


Abbildung 1: Von LTA-1 katalysierte Reaktion von Glycin und Bicyclo[2.2.1]hept-5-en-2-carbaldehyd zu Norl

Ziel der Zusammenarbeit ist es, einen im Industriemaßstab anwendbaren Produktionsprozess zu entwickeln, bei dem die Prozessparameter so adjustiert werden, dass eine hohe Produktivität aktiver L-Threonin Aldolase erreicht wird.

Entwicklung bis zur Zusammenarbeit

Das LTA-1-Gen aus *P. putida* wurde in ein pQE-Plasmid (Qiagen, USA) kloniert und mit diesem in *E. coli* BL21 exprimiert. Die Expression des LTA-1 Gens kann durch eine T5-Promotersequenz sowohl mit IPTG als auch mit Laktose induziert werden. Weiters verfügt das Plasmid über eine Ampicillinresistenz und einen $laqI^{Q}$-Repressor.

Der Nachweis von L-TA1 erfolgt über die vom Enzym katalysierte Reaktion von DL-Phenylserin zu Glycin und Benzaldehyd (Abbildung 2), welches photometrisch bei 300 nm detektiert wird. Ein Assay für diesen Nachweis wurde entwickelt. Die Steigung des anfänglich linearen Bereichs der Absorptionskurve bei 300 nm gilt als Substratumsatzgeschwindigkeit und kann somit über den Extinktionskoeffizienten von Benzaldehyd, der für dieses Assayvolumen spezifisch ist, für die Berechnung der Enzymaktivität herangezogen werden.

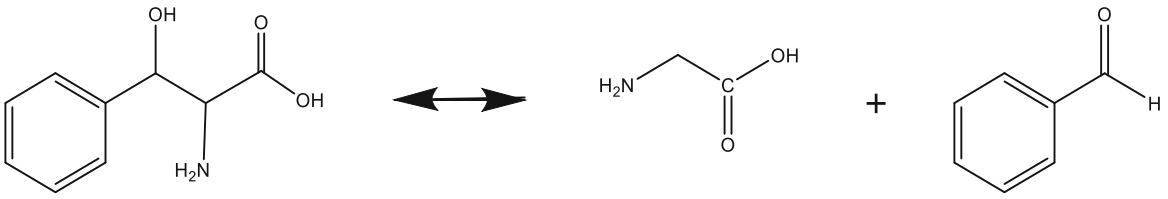


Abbildung 2: Spaltungsreaktion von DL-Phenylserin, katalysiert von LTA-1
Auf dieser Reaktion basiert der enzymatische Assay. Benzaldehyd wird bei 300 nm im Reaktionsgemisch photometrisch detektiert.

Erste Kultivierungsvorversuche in Schüttelflaschen im Maßstab von 100 mL wurden mit einer durch Hausjell et al. [33] beschriebene Modifikation des von DeLisa et al. [44] entwickelten Minimalmediums durchgeführt. Dabei wurden entweder Glucose, Glycerin oder die von Studier et al. [59] beschriebene Mischung 5052, bestehend aus Glucose, Glycerin und Laktose, als Kohlenstoffquelle verwendet. Als Induktionssubstanzen wurden Isopropyl-β-D-thiogalactopyranosid (IPTG) oder Laktose verwendet. Das vielversprechendste Ergebnis im Hinblick auf Enzymaktivität von LTA-1 lieferte die Variante Delisa+5052.

Durchgeführte Arbeiten im Zuge der Zusammenarbeit

Upstream Processing

Um einen industriellen Produktionsprozess für LTA-1 zu entwickeln, wurden verschiedene Induktionsbedingungen in kleinem Maßstab getestet. Dafür wurden zwei Mal vier Fermentationsexperimente in DASBox Bioreaktoren (Eppendorf, Hamburg, Deutschland) mit 250 mL Arbeitsvolumen durchgeführt. Vier dieser Experimente wurden mit IPTG als Induktionssubstanz durchgeführt, weitere vier mit Laktose.

Für jeweils vier Fermentationen wurden während der Induktionsphase unterschiedliche spezifische Wachstumsraten (μ) und Temperaturen (T) eingestellt, um die optimalen Bedingungen für die Expression von aktiver LTA-1 auszuloten. Als Medium wurde das von DeLisa et al. [44] beschriebene Minimalmedium verwendet.

Die Fermentationsexperimente bestanden aus drei Phasen. In der ersten Phase wurde die Fermentation im Batch durchgeführt, um die Biomassekonzentration auf etwa 8 g/L zu bringen. Die Temperatur wurde auf 37 °C eingestellt, der pH-Wert auf 7. Nach vollständigem Verbrauch der Kohlenstoffquelle im Medium wurde in der zweiten Phase des Prozesses ein Fed Batch gestartet, um die Biomassekonzentration weiter auf etwa 30 g/L anwachsen zu lassen, mit gleichen Temperatur- und pH-Einstellungen. Die letzte Phase bestand ebenso aus einem Fed Batch, allerdings mit anfänglicher Zugabe von Induktionssubstanz. In vier dieser acht Fermentationen wurde die Expression von LTA-1 mit einer einmaligen pulsartigen Zugabe von IPTG induziert, in weiteren vier erfolgte die Induktion mit einer ebenfalls pulsartigen Zugabe von Laktose, begleitet von einer fortlaufenden Zugabe über den Feedzulauf bis zum Fermentationsende. Der pH-Wert wurde auf 7,1 erhöht. Die Temperatur und μ wurden gemäß

Tabelle 1 eingestellt. Die Regelung von μ erfolgte über die Feedzulaufrate, da μ mit der spezifischen Substrataufnahmerate q_s über den Ausbeutekoeffizient $Y_{x/s}$ mathematisch zusammenhängt. Der Induktionszeitraum betrug bei allen Fermentationen zwölf Stunden. Probenahmen erfolgten alle zwei Stunden.

Mit den entnommenen Proben wurde die optische Dichte bei 600 nm (OD_{600}) und der Proteingehalt im Medium photometrisch bestimmt. Die Messung der Zuckerkonzentration im Medium erfolgte über Hochdruckflüssigkeitschromatographie (HPLC). Außerdem wurde die Trockenbiomasse bestimmt.

Aus den acht Fermentationsläufen im kleinen Maßstab wurde anschließend eine vielversprechende Variante ausgewählt und ein Fermentationslauf in einem Sartorius C Reaktor mit 20 L Arbeitsvolumen unter denselben Induktionsbedingungen durchgeführt.

Die Parameter aller Fermentationen sind in Tabelle 1 angeführt.

Tabelle 1: Phasen und Parameter der Fermentationsexperimente
Bei der Glucosekonzentration im Batch handelt es sich um jene im Reaktor, im Fed Batch um jene im Zulauf.

	pH		T [°C]	μ [h^{-1}]	$c_{Glucose}$ [g/L]	Induktionsvariante	
Batch	7.1		37	μ_{max}	20	IPTG	Laktose
Fed Batch	7.1		37	0.1	200		
Induzierter Fed Batch	7	Fermentation 1	30	0.1	200	Puls auf 0.1 mM	Puls auf 20 g/L, 150 g/L im Zulauf
		Fermentation 2	25	0.05			
		Fermentation 3	25	0.1			
		Fermentation 4	30	0.05			
		Scale up	30	0.05			

Downstream Processing

Sämtliche Produktanalytik wurde von Valanx Biotech selbst durchgeführt.

Bei jeder Probenahme wurden Biomassepellets für die spätere Aufarbeitung eingefroren. Diese Proben wurden für die Messung der Enzymaktivität chemisch aufgeschlossen. Die Aktivitätsbestimmung erfolgte über den oben beschriebenen Assay.

Die gesamte durch Zentrifugation geerntete Biomasse von jedem Fermentationsexperiment wurde ebenfalls für den späteren Aufschluss im größeren Maßstab eingefroren. Diese kann für zukünftige Synthesen der Aminosäure Norl verwendet werden.
Da für die Synthese von Norl lediglich das rohe Lysat eingesetzt wird, ist keine weitere Aufreinigung von LTA-1 notwendig.

Zusammenfassung der Ergebnisse

Physiologie

Die in Tabelle 2 bis Tabelle 10 angeführten physiologischen Daten der Fermentationsläufe weisen schließende C-Bilanzen auf. Dies lässt auf eine korrekte Datenauswertung schließen.

Betrachtet man die über Trockenbiomasse und OD_{600} bestimmten spezifischen Wachstumsraten bei den Fermentationen mit IPTG-Induktion und vergleicht diese mit den eingestellten Parametern, so sieht man, dass die Abweichung bis hin zu den letzten Probenahmezeitpunkten recht gering ist. Dies bedeutet, dass die angenommene Biomasseausbeute mit der Prozessführung übereinstimmt und das Zellwachstum bis zum Ende stabil bleibt. Bei der Laktoseinduktion hingegen beobachtet man gegen Ende der Fermentation eine Abnahme der spezifischen Wachstumsraten. Ebenso sieht man nach der Induktion mit dem Laktosepuls in den ersten vier bis sechs Stunden einen Anstieg der spezifischen Laktoseaufnahmerate und eine anschließende Abnahme. Dies deutet darauf hin, dass sich die Zellen erst für Laktoseaufnahme adaptieren müssen, bis schließlich bei den zwei Läufen mit $\mu = 0.1\ h^{-1}$ Laktose im Medium akkumuliert, was osmotischen Stress für die Zellen verursacht.

Durch den Scale-Up-Versuch, bei dem die Induktion mit IPTG mit den Parametern $\mu = 0.05\ h^{-1}$ und $T = 30\ °C$ verlaufen ist, konnten die physiologischen Daten des entsprechenden Versuchs im kleinen Maßstab reproduziert werden, wie in Tabelle 10 gezeigt.

Produktbildung

In Abbildung 3 ist die volumetrische Enzymaktivität im Fermentationsverlauf dargestellt.

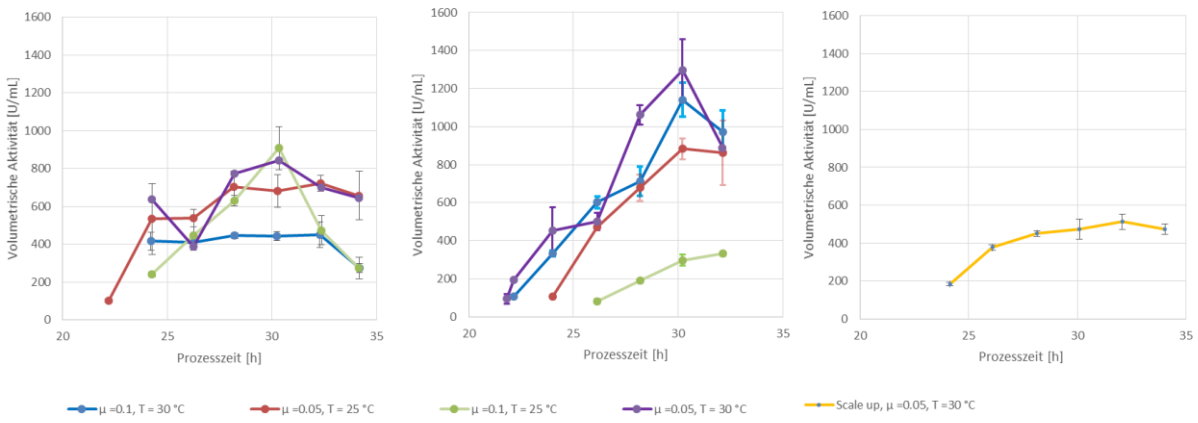


Abbildung 3: Volumetrische Enzymaktivität, bezogen auf die Fermentationsbrühe
Auf dem linken Graphen befinden sich die volumetrischen Enzymaktivitäten der vier Fermentationen mit IPTG-Induktion. Der mittlere Graph zeigt die Laktose-Induktion. Rechterhand befindet sich das Scale-up, bei dem mit IPTG induziert wurde. Die Induktion erfolgte nach 22 h Prozesszeit.

Bei allen Fermentationen scheint eine niedrigere Wachstumsrate während der Induktion vorteilhaft für die Produktbildung zu sein. Da bei dem Experiment im kleinen Maßstab mit den Induktionsparametern $\mu = 0.05\ h^{-1}$ und T = 30 °C die volumetrische Produktaktivität bis zum Ende der Induktion stabil bleibt, und weil sich eine Induktion mit IPTG einfacher in einem größeren Maßstab realisieren lässt, wurden diese Induktionsparameter für das Scale-up-Experiment verwendet.

In Abbildung 4 ist die biomassespezifische Aktivität der jeweils vielversprechendsten Fermentationen der zwei Induktionsvarianten sowie jene des Schüttelkolbenvorversuchs mit Delisa+5052 dargestellt. Die biomassespezifische Aktivität bezieht sich auf die Aktivität jenes Lysats, das aus 1 mL Kulturbrühe mit $OD_{600} = 0.5$ entstammt.

Für zukünftige Fermentationen im großen Maßstab für die Produktion von aktivem LTA-1 wird folgende Strategie zur Anwendung kommen:

Als Induktor wird Laktose verwendet werden, mit denselben Induktionsparametern wie jene, die bei dem durchgeführten Scale-up-Experiment eingestellt wurden ($\mu = 0.05\ h^{-1}$ und T = 30 °C), allerdings mit einem Induktionszeitraum von acht bis zehn Stunden. Für die erwartete Steigerung der Ausbeute wird der aufwändigere Prozessaufbau in Kauf genommen. Anstatt Glucose soll Glycerin als Kohlenstoffquelle verwendet werden, da hier die Katabolitrepression zu Laktose nicht so stark ausgeprägt ist wie im Falle der Glukose. Diese Herangehensweise wird durch die Daten des Vorexperiments in Schüttelflaschen mit DeLisa+5052 gestützt, wie in Abbildung 5 dargestellt.

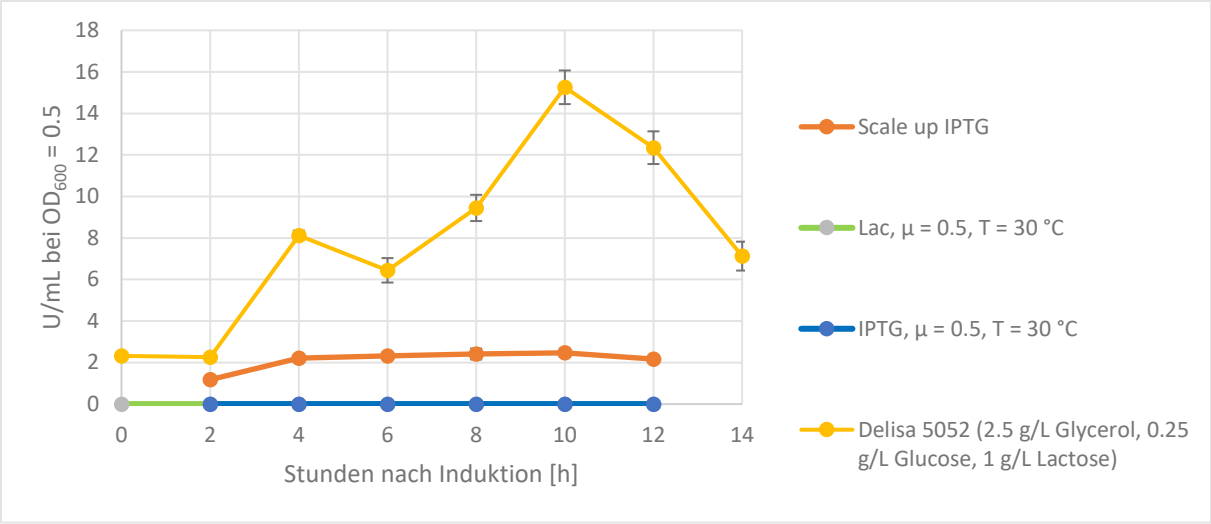


Abbildung 4: Spezifische Enzymaktivität über den Zeitverlauf der Induktion
Die auf OD_{600} = 0.5 normierte Enzymaktivität des Schüttelflaschenexperiments mit DeLisa+5052 zeigt die höchsten Werte nach 10 h. Da die Kohlenstoffquellenmischung in diesem Medium in einer Schüttelflasche als Autoinduktionsmedium fungiert, konnte nur abgeschätzt werden, wann die Induktion mit Laktose tatsächlich beginnt. Messungen des Laktosegehalts der Fermentationsbrühe mittels HPLC haben ergeben, dass beim ersten Datenpunkt tatsächlich fast die gesamte Laktose im Medium noch vorhanden ist, also hat die Induktion erst kürzlich vorher eingesetzt.

Nutzen für das Unternehmen

Produktionsprozess etabliert

Ein Produktionsprozess für das Enzym L-TA1 wurde entwickelt. Dieser ist mit kostengünstigen Medien bzw. Induktionsvarianten realisierbar und im Maßstab auf eine industrielle Produktion vergrößerbar.

Publizierbare Ergebnisse

Dieser Prozess ist der erste bekannte Produktionsprozess in *E. coli* für LTA-1. Überexpression von L-TA1 in *E. coli* wurde erstmals gezeigt. Diese neuen wissenschaftlichen Erkenntnisse könnten im Rahmen einer Publikation veröffentlicht werden.

Synthese von Norl ermöglicht

Der entwickelte Prozess liefert große Mengen aktive LTA-1, welche für die Synthese der Aminosäure Norl essentiell ist. Diese Aminosäure kann somit ebenso in einem großtechnischen Produktionsprozess hergestellt werden, da die Versorgung mit dem notwendigen Enzym gewährleistet ist.

Anhang

Tabelle 2: IPTG-Induktion 1, μ = 0.1 1/h, T = 30 °C

Phase	Prozesszeit	qs Gluc	μ	C-balance
-	[h]	[g/g/h]	[h^{-1}]	-
Batch End	6.0	0.609 ± 0.044	0.304 ± 0.008	1.067 ± 0.355
FB End, Induc	22.1	0.244 ± 0.012	0.100 ± 0.001	0.938 ± 0.259
Induction	24.2	0.303 ± 0.016	0.119 ± 0.008	0.906 ± 0.062
Induction	26.3	0.285 ± 0.015	0.127 ± 0.010	0.952 ± 0.060
Induction	28.2	0.288 ± 0.015	0.091 ± 0.016	0.846 ± 0.094
Induction	30.2	0.286 ± 0.015	0.101 ± 0.019	0.943 ± 0.096
Induction	32.3	0.278 ± 0.016	0.065 ± 0.014	0.894 ± 0.086
Induction	34.2	0.242 ± 0.017	0.030 ± 0.008	0.833 ± 0.075

Tabelle 3: IPTG-Induktion 2, μ = 0.05 1/h, T = 25 °C

Phase	Prozesszeit	qs Gluc	μ	C-balance
-	[h]	[g/g/h]	[h^{-1}]	-
Batch End	6.1	0.538 ± 0.045	0.282 ± 0.005	1.116 ± 0.374
FB End, Induc	22.2	0.245 ± 0.013	0.097 ± 0.002	0.928 ± 0.262
Induction	24.3	0.150 ± 0.008	0.023 ± 0.010	0.715 ± 0.236
Induction	26.3	0.145 ± 0.008	0.074 ± 0.015	1.059 ± 0.139
Induction	28.2	0.142 ± 0.008	0.036 ± 0.031	0.765 ± 0.275
Induction	30.3	0.145 ± 0.008	0.053 ± 0.027	1.003 ± 0.226
Induction	32.3	0.147 ± 0.007	0.044 ± 0.009	1.027 ± 0.100
Induction	34.2	0.153 ± 0.008	0.020 ± 0.009	0.805 ± 0.097

Tabelle 4: IPTG-Induktion 3, μ = 0.1 1/h, T = 25 °C

Phase	Prozesszeit	qs Gluc	μ	C-balance
-	[h]	[g/g/h]	[h^{-1}]	-
Batch End	6.1	0.628 ± 0.056	0.298 ± 0.024	1.141 ± 0.432
FB End, Induc	22.1	0.273 ± 0.022	0.099 ± 0.002	1.089 ± 0.446
Induction	24.3	0.311 ± 0.020	0.115 ± 0.033	1.021 ± 0.242
Induction	263	0.304 ± 0.016	0.139 ± 0.015	0.983 ± 0.085
Induction	28.2	0.326 ± 0.017	0.117 ± 0.024	0.847 ± 0.101
Induction	30.3	0.294 ± 0.016	0.113 ± 0.019	0.932 ± 0.088
Induction	32.4	0.243 ± 0.016	0.071 ± 0.013	0.940 ± 0.101
Induction	34.2	0.219 ± 0.018	0.022 ± 0.014	0.821 ± 0.101

Tabelle 5: IPTG-Induktion 4, μ = 0.05 1/h, T = 30 °C

Phase	Prozesszeit	qs Gluc	μ	C-balance
-	[h]	[g/g/h]	[h^{-1}]	-
Batch End	6.1	0.761 ± 0.069	0.313 ± 0.023	0.910 ± 0.319
FB End, Induc	22.1	0.264 ± 0.014	0.101 ± 0.002	0.885 ± 0.244
Induction	24.3	0.161 ± 0.008	0.054 ± 0.013	0.922 ± 0.215
Induction	26.3	0.152 ± 0.008	0.070 ± 0.013	0.985 ± 0.108
Induction	28.2	0.149 ± 0.008	0.043 ± 0.009	0.765 ± 0.083
Induction	30.3	0.148 ± 0.008	0.062 ± 0.009	0.932 ± 0.083
Induction	32.4	0.140 ± 0.007	0.060 ± 0.009	0.985 ± 0.099
Induction	34.2	0.142 ± 0.007	0.045 ± 0.014	0.863 ± 0.135

Tabelle 6: Laktose-Induktion, μ = 0.1 1/h, T = 30 °C

Phase	Prozesszeit	qs Gluc	qs Lac	μ	C-balance
-	[h]	[g/g/h]	[g/g/h]	[h^{-1}]	-
Batch End	6.5	0.627 ± 0.036	-	0.267 ± 0.011	0.943 ± 0.365
FB End	21.8	0.244 ± 0.013	-	0.091 ± 0.001	0.908 ± 0.260
Lac Pulse	22.1	0.525 ± 0.027	-	-0.083 ± 0.119	-0.096 ± 0.153
Induction	23.9	0.232 ± 0.013	0.113 ± 0.011	0.154 ± 0.010	1.099 ± 0.103
Induction	26.1	0.231 ± 0.012	0.265 ± 0.011	0.107 ± 0.009	0.793 ± 0.067
Induction	28.1	0.252 ± 0.012	0.382 ± 0.015	0.105 ± 0.014	0.700 ± 0.073
Induction	30.1	0.219 ± 0.012	0.101 ± 0.010	0.116 ± 0.013	1.202 ± 0.099
Induction	32.0	0.225 ± 0.012	0.087 ± 0.010	0.120 ± 0.012	1.204 ± 0.074
Induction	34.0	0.247 ± 0.012	0.014 ± 0.013	0.067 ± 0.018	0.942 ± 0.098

Tabelle 7: Laktose-Induktion, μ = 0.05 1/h, T = 25 °C

Phase	Prozesszeit	qs Gluc	qs Lac	μ	C-balance
-	[h]	[g/g/h]	[g/g/h]	[h-1]	-
Batch End	6.5	0.617 ± 0.041	-	0.281 ± 0.020	0.980 ± 0.362
FB End	21.8	0.241 ± 0.013	-	0.089 ± 0.001	0.892 ± 0.249
Lac Pulse	22.1	0.486 ± 0.027	-	-0.304 ± 0.102	0.138 ± 0.098
Induction	23.9	0.119 ± 0.006	0.088 ± 0.005	0.080 ± 0.010	1.011 ± 0.104
Induction	26.1	0.109 ± 0.006	0.111 ± 0.005	0.095 ± 0.011	1.163 ± 0.101
Induction	28.1	0.099 ± 0.006	0.229 ± 0.009	0.087 ± 0.016	0.853 ± 0.101
Induction	30.1	0.103 ± 0.005	0.113 ± 0.005	0.081 ± 0.017	1.109 ± 0.134
Induction	32.0	0.094 ± 0.005	0.090 ± 0.004	0.069 ± 0.013	1.163 ± 0.134
Induction	34.0	0.103 ± 0.005	0.091 ± 0.004	0.038 ± 0.019	0.832 ± 0.167

Tabelle 8: Laktose-Induktion 3, μ = 0.1 1/h, T = 25 °C

Phase	Prozesszeit	qs Gluc	qs Lac	μ	C-balance
-	[h]	[g/g/h]	[g/g/h]	[h-1]	-
Batch End	6.5	0.716 ± 0.053	-	0.317 ± 0.027	0.944 ± 0.347
FB End	21.9	0.254 ± 0.015	-	0.090 ± 0.002	0.880 ± 0.247
Lac Pulse	22.1	0.690 ± 0.030	-	-0.474 ± 0.358	-0.154 ± 0.179
Induction	23.9	0.245 ± 0.013	0.000 ± 0.016	0.145 ± 0.010	1.487 ± 0.115
Induction	26.1	0.233 ± 0.013	0.007 ± 0.013	0.148 ± 0.020	1.256 ± 0.139
Induction	28.1	0.208 ± 0.012	0.133 ± 0.009	0.163 ± 0.024	1.144 ± 0.113
Induction	30.1	0.205 ± 0.011	0.087 ± 0.009	0.096 ± 0.018	0.983 ± 0.117
Induction	32.0	0.177 ± 0.011	0.024 ± 0.011	0.078 ± 0.017	1.205 ± 0.118
Induction	34.0	0.281 ± 0.013	-0.003 ± 0.014	-0.002 ± 0.014	0.392 ± 0.064

Tabelle 9: Laktose-Induktion 4, μ = 0.05 1/h, T = 30 °C

Phase	Prozesszeit	qs Gluc	qs Lac	μ	C-balance
-	[h]	[g/g/h]	[g/g/h]	[h-1]	-
Batch End	6.5	0.625 ± 0.035	-	0.260 ± 0.004	0.914 ± 0.330
FB End	21.9	0.246 ± 0.013	-	0.089 ± 0.001	0.883 ± 0.257
Lac Pulse	22.1	0.521 ± 0.028	-	-0.687 ± 0.152	0.234 ± 0.071
Induction	23.9	0.115 ± 0.006	0.188 ± 0.007	0.143 ± 0.009	1.283 ± 0.093
Induction	26.1	0.104 ± 0.005	0.280 ± 0.011	0.076 ± 0.012	0.796 ± 0.069
Induction	28.2	0.092 ± 0.005	0.091 ± 0.004	0.070 ± 0.011	1.207 ± 0.106
Induction	30.1	0.107 ± 0.005	0.089 ± 0.004	0.054 ± 0.014	0.927 ± 0.128
Induction	32.0	0.094 ± 0.005	0.074 ± 0.004	0.063 ± 0.018	1.191 ± 0.175
Induction	34.0	0.094 ± 0.005	0.076 ± 0.004	0.034 ± 0.011	0.924 ± 0.112

Tabelle 10: Scale-up, IPTG Induktion, μ = 0.05 1/h, T = 30 °

Phase	Prozesszeit	qs Gluc	μ	C-balance
-	[h]	[g/g/h]	[h-1]	-
Batch End	6.1	0.573 ± 0.029	0.235 ± 0.001	0.872 ± 0.343
FB End, Induc	22.1	0.225 ± 0.012	0.090 ± 0.001	0.962 ± 0.205
Induction	24.3	0.136 ± 0.007	0.054 ± 0.016	1.002 ± 0.153
Induction	26.3	0.135 ± 0.007	0.056 ± 0.013	0.992 ± 0.114
Induction	28.2	0.133 ± 0.010	0.054 ± 0.049	0.982 ± 0.466
Induction	30.3	0.133 ± 0.009	0.055 ± 0.053	0.937 ± 0.530
Induction	32.4	0.133 ± 0.007	0.046 ± 0.016	0.926 ± 0.146
Induction	34.2	0.136 ± 0.007	0.031 ± 0.020	0.781 ± 0.186

Literaturverzeichnis

[1] J. Steinreiber, K. Fesko, C. Reisinger, M. Schürmann, F. van Assema, M. Wolberg, D. Mink, H. Griengl, Threonine aldolases-an emerging tool for organic synthesis, Tetrahedron. 63 (2007) 918–926. https://doi.org/10.1016/j.tet.2006.11.035.

[2] K. Fesko, M. Uhl, J. Steinreiber, K. Gruber, H. Griengl, Biocatalytic access to a,a-dialkyl-a-amino acids by a mechanism-based approach, Angew. Chemie - Int. Ed. 49 (2010) 121–124. https://doi.org/10.1002/anie.200904395.

[3] N. Dückers, K. Baer, S. Simon, H. Gröger, W. Hummel, Threonine aldolases-screening, properties and applications in the synthesis of non-proteinogenic β-hydroxy-α-amino acids, Appl. Microbiol. Biotechnol. 88 (2010) 409–424. https://doi.org/10.1007/s00253-010-2751-8.

[4] J. Hausjell, J. Weissensteiner, C. Molitor, H. Halbwirth, O. Spadiut, E. coli HMS174(DE3) is a sustainable alternative to BL21(DE3), Microb. Cell Fact. 17 (2018) 169. https://doi.org/10.1186/s12934-018-1016-6.

[5] M.P. DeLisa, J. Li, G. Rao, W.A. Weigand, W.E. Bentley, Monitoring GFP-operon fusion protein expression during high cell density cultivation ofEscherichia coli using an on-line optical sensor, Biotechnol. Bioeng. 65 (1999) 54–64. https://doi.org/10.1002/(SICI)1097-0290(19991005)65:1<54::AID-BIT7>3.0.CO;2-R.

[6] F.W. Studier, Protein production by auto-induction in high-density shaking cultures, Protein Expr. Purif. 41 (2005) 207–234. https://doi.org/10.1016/J.PEP.2005.01.016.

Conclusion of the thesis

Following hypotheses were formulated.

Active soluble LTA-1 expression and the productivity of the expression system for this enzyme is dependent on

(1) the type of inducer (IPTG and lactose, respectively).
(2) the temperature during the induced fed-batch.
(3) the substrate feeding rate during the induced fed-batch.

As elaborated in this thesis, it was shown that (1) the type of inducer, (2) the temperature during the induced fed-batch and (3) the substrate feeding rate during the induced fed-batch have a substantial influence on active and soluble LTA-1 expression and the productivity of this expression system for this particular enzyme. This was shown with respect to biomass-specific enzyme activity, productivity, as well as inclusion body formation, while also shedding light on the physiological influence of these conditions on the production host. Additionally, the results were interpreted in light of the unconventional expression host E. coli BL21 with the target protein expressed under control of the T5 promoter, and its counterplay with highly expressed LacI.

In the paper draft, it was concluded that following conditions yield the most promising results regarding target protein expression in this host:

- μ_{ind} = 0.05 h^{-1} & T_{ind} = 30 °C
- Lactose induction for 6-8 h.

Following goal was achieved.

Multiple LTA-1 expression conditions in the chosen production host were investigated to find one suitable for scale-up, to be used for further fermentations with the aim of LTA-1 production for use as biocatalyst in the synthesis of NorI.

Due to ease of operation and uncertainty regarding the results at this point of the practical work of the thesis, following conditions were chosen for the scale-up experiment:

- μ_{ind} = 0.05 h^{-1} & T_{ind} = 30 °C
- IPTG induction

The cells of the scale up experiment were given to Valanx Biotech after harvest for homogenization to be further processed and used in NorI synthesis.